

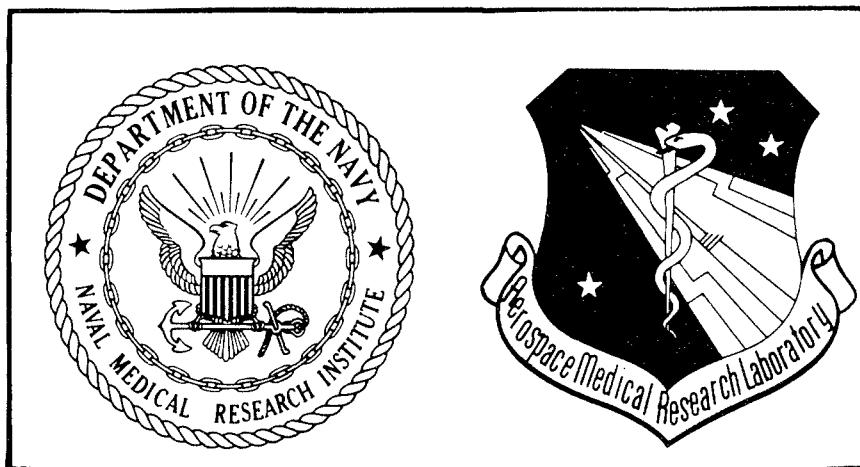
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**PROCEEDINGS OF THE TWELFTH CONFERENCE ON
ENVIRONMENTAL TOXICOLOGY 3, 4, AND 5 NOVEMBER 1981**

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<p>Papers were presented in the area of animal modeling of carcinogenesis, sex-related factors influencing toxicity, use of experimental toxicologic data in estimating occupational hazards, use of physiologic kinetics in toxicology, and effects of toxic substances on aquatic organisms.</p>														

PREFACE

The Twelfth Conference on Environmental Toxicology was held in Dayton, Ohio on 3, 4, and 5 November 1981. Sponsor was the University of California, Irvine under the terms of Contract No. F33615-80-C-0512, Work Unit 63020115 with the Aerospace Medical Research Laboratory, Aerospace Medical Division, Air Force Systems Command, Wright-Patterson Air Force Base, Ohio, partially funded by the U. S. Naval Medical Research Institute, Toxicology Detachment, Wright-Patterson Air Force Base, Ohio under MIPR No. N6433482MP00009.

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TABLE OF CONTENTS

	<u>Page</u>
WELCOMING REMARKS.....	7
Anthony A. Thomas, M.D.	
INTRODUCTORY ADDRESS.....	9
Lutz A. Kiesow, M.D., Ph.D.	
SESSION I - ANIMAL MODELING OF CARCINOGENESIS.....	12
1 - MECHANISMS OF ASBESTOS-INDUCED CARCINOGENESIS IN HAMSTER TRACHEA.....	13
Brooke T. Mossman, Ph.D.	
2 - CHEMICAL CARCINOGENESIS IN CULTURED RAT TRACHEAL EPITHELIUM.....	24
Vernon E. Steele, Ph.D.	
3 - A MOUSE LUNG CARCINOMA MODEL SYSTEM.....	35
Richard E. Kouri, Ph.D.	
4 - STRAIN A MOUSE LUNG ADENOMA BIOASSAY FOR CHEMICAL CARCINOGENS.....	50
Gary D. Stoner, Ph.D.	
5 - METABOLISM OF BENZO(A)PYRENE IN CULTURED HUMAN BRONCHUS.....	68
Gary D. Stoner, Ph.D.	
SESSION II - THE IMPORTANCE OF SEX-SPECIFIC EFFECTS IN TOXICOLOGY.....	95
6 - ORGANIZATIONAL EFFECTS OF HORMONES AND HORMONALLY-ACTIVE XENOBIOTICS ON POSTNATAL DEVELOPMENT.....	96
Coral A. Lamartiniere, Ph.D.	
7 - MALE vs. FEMALE MEDIATED TERATOGENESIS.....	122
Jeanne M. Manson, Ph.D.	
8 - WHOLE ANIMAL SYSTEMS FOR MEASURING CHEMICALLY INDUCED GENOTOXICITY.....	140
Byron E. Butterworth, Ph.D.	
9 - TOXICITY, GENDER, AND NEUROBEHAVIORAL RESPONSES.....	147
Joel L. Mattsson, D.V.M., Ph.D.	

TABLE OF CONTENTS

	<u>Page</u>
SESSION III - THE APPLICATION OF TOXICOLOGY DATA TO HAZARD ASSESSMENT.....	160
10 - AN OVERVIEW..... Col. Gale D. Taylor, USAF, BSC	161
11 - AN INDUSTRIAL HYGIENIST'S VIEW: RELATIONSHIPS BETWEEN INDUSTRIAL HYGIENE SAMPLING, HUMAN EXPOSURE STANDARDS, AND EXPERIMENTAL TOXICOLOGY..... Lt. Col. James C. Rock, USAF, BSC	164
12 - EVALUATING HEALTH HAZARDS ASSOCIATED WITH AIRCRAFT FUEL CELL MAINTENANCE..... Capt. Edward C. Bishop, USAF, BSC	192
13 - A SUGGESTED WORKPLACE MONITORING PROGRAM FOR MANAGING THE OCCUPATIONAL EXPOSURE OF U.S. AIR FORCE EMPLOYEES..... Capt. Peter A. Lurker, USAF, BSC	203
14 - THE DEVELOPMENT OF A COMPUTERIZED DATA BASE TO ENHANCE APPLIED AND THEORETICAL TOXICOLOGY..... Maj. Robert A. Lombard, Jr., USAF, BSC	221
SESSION IV - APPLICATIONS OF PHYSIOLOGICAL KINETIC MODELING IN TOXICOLOGY.....	233
15 - PHARMACOKINETIC EVALUATIONS IN RELATION TO USE OF TOXICOLOGICAL DATA..... John C. Ramsey, Ph.D.	234
16 - A PHYSIOLOGICALLY-BASED PHARMACOKINETIC MODEL FOR 2,5-HEXANEDIONE..... Michael J. Angelo, Ph.D.	250
17 - PHYSIOLOGICAL MODELING OF ENTERIC TRANSPORT..... Peter M. Bungay, Ph.D.	287
18 - PHYSIOLOGICAL PHARMACOKINETIC MODELS FOR THE INHALATION OF GASES AND VAPORS..... Melvin E. Andersen, Ph.D.	299

TABLE OF CONTENTS

	<u>Page</u>
SESSION V - ENVIRONMENTAL EFFECTS.....	321
19 - ASSESSING THE HAZARD OF CHEMICALS TO AQUATIC LIFE: A PERSPECTIVE ON THE STATE-OF- THE-ART.....	322
William E. Bishop, Ph.D.	
20 - BEHAVIOR OF HYDROCARBON FUELS IN AQUATIC SYSTEMS.....	336
James H. Smith, Ph.D.	
21 - MICROBIAL DEGRADATION OF XENOBIOTIC COMPOUNDS.....	354
Al W. Bourquin, Ph.D.	
22 - UTILIZATION OF AQUATIC ORGANISMS FOR CONTINUOUSLY MONITORING THE TOXICITY OF INDUSTRIAL WASTE EFFLUENTS.....	370
William H. van der Schalie, Ph.D.	
23 - ULTRASTRUCTURAL ALTERATIONS IN FATHEAD MINNOWS AFTER EXPOSURE TO JP-4.....	393
William N. Norton, Ph.D.	

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WELCOMING REMARKS

Anthony A. Thomas, M.D.
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"In the beginning, it was customary to use a rat or two, an odd rabbit, and a few mice. Before long it was clear that toxicity in man could not be readily predicted in this way. So, the number of rats increased, and before long someone started statistics, so the number of rats increased still further. Dogs came in. Rabbits went out. Cats became scarce. Now this latter is no joke because during the war, back in the early 40's, I used to meet a not-too-pleasant individual under the Brooklyn Bridge at midnight where I exchanged money for cats.

Well, predictions improved but still there was a long way to go. So the number of rats increased, so did the dogs. So did the mice. More species were added--monkeys, chimps, marmosets, quail, frogs and pigs. Longer tests were required, ten days, two weeks, six months, two years, to one life span. Still no closer to predictability in man. Once it was just toxicity, and then it was multigeneration tests, carcinogens came in, then co-carcinogens, and if you couldn't find a carcinogen, then you looked for a mutagen. If you couldn't find a mutagen, then you looked for a teratogen. We used not one species but many species. Not one strain, but many strains. Outbred. Inbred. Brother-sister mated. Random mated. Still no better predictability. Once you counted just the dead. This procedure was charged with fallacy, so everything that could be weighed was weighed, and everything that could be removed was sliced and examined histologically. The function of every organ was looked into. From the cellular, we went to the sub-cellular. Radio isotopes became a must. Physiology gave way to psychology. And now not even the rat doubts the results."⁽¹⁾

In closing, allow me to quote Harvey Cushing, who has so beautifully expressed the true need for personal communication and gatherings among scientists in his book titled "The Life of Sir William Osler":

"By no means the smallest advantage of our meetings is the promotion of harmony and good-fellowship. Medical men, particularly in smaller places, live too much apart and do not see enough of each other. In large cities we run each other's angles down and carom off each other without feeling the shock very much, but it is an unfortunate circumstance that in many towns, the friction being

(1) H. W. Hays, Problems in the Interpretation and Extrapolation of Animal Data to Man.

a small surface, hurts and mutual misunderstandings arise to the destruction of all harmony. As a result of this may come a professional isolation with a corroding influence of a most disastrous nature, converting a genial, good fellow in a few years into a bitter old Timon, railing against the practice of medicine in general and his colleagues in particular. As a preventive of such a malady, attendance upon our annual gatherings is absolute, as a cure it is specific. But I need not dwell on this point--he must indeed be a stranger in such meetings as ours who has not felt the glow of sympathy and affection as the hand of a brother worker has been grasped in kindly fellowship."

INTRODUCTORY ADDRESS

Lutz A. Kiesow, M.D., Ph.D.

Naval Medical Research Institute
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Bethesda, Maryland

Ladies and Gentlemen, it is a pleasure to be here this morning and to welcome you to the Twelfth Conference on Environmental Toxicology. At the same time, I would like to thank the organizers for inviting me not only to participate in this Conference, but also to be its Chairman. This invitation, however, left one question for me to ponder and, since it was an early invitation, I could really do so for some time. The question was: Why would nearly one hundred environmental toxicologists invite one biochemist to their conference?

The first and obvious answer that comes to mind is--they want to get even! On second thought, however, there may have been more to this act than is revealed by a superficial glance, and it is this thought that I would like to carry a little further with you this morning.

When Prometheus stole the fire from the gods to give it to man, he himself could have easily become the founder of environmental toxicology and, for that matter, the first toxicologist. No doubt, either he or the first man who took over this fire must have used some combustible matter to maintain an oxidation process which most likely was chemically incomplete and produced reaction products other than H_2O and CO_2 . While mythology remains vague with regard to Prometheus' interests in combustion processes and chemical oxidations, man's interest was undoubtedly stimulated to the point that he decided to embark immediately on an intense pursuit of the process and its consequences, a decision which led to, as we in the Government and DOD would have called it, a long-lasting RDT & E effort.

I am recalling these perhaps not-so-well documented events only to remind you that it was man's own, yet early and firm, decision to advance technologic development rapidly to a point where his own life and his own and various forms of civilizations have become almost completely dependent upon technology. As an inseparable part of this dependence, however, man now must also accept not only the benefits but all other consequences of these technologic advances. One such consequence is the ever-growing number of xenobiotics that modern technology creates and requires.

Well then, it was xenobiotics, the non-biologic substances totally foreign to living organisms, that Prometheus produced with his fire, and it is xenobiotics and associated problems that bring toxicologists together to a conference on environmental toxicology. Most important for me, it is those xenobiotics that give a biochemist like me a certain feeling of security at such gatherings. Let me explain why this is so.

First of all, I am firmly convinced that exposure to and incorporation of xenobiotics has become part of one's life, not only in the military and despite the concerns of environmentalists, for they too use xenobiotic drugs, drive automobiles, fly in airplanes, eat fertilizer-dependent foods and house and dress----in other words, they depend as completely on modern technology as you and I.

But this conviction of mine has other consequences as well. If we have to accept xenobiotics as a part of our environment, toxicology in general, and environmental toxicology, in particular, face rapidly expanding tasks. We can no longer be satisfied with determining dose-response relationships and establishing exposure limits of an ever-growing number of pesticides, solvents, occupationally used compounds, drugs, food additives, stabilizers, antioxidants, fuels, and many other compounds. We must realize that xenobiotics, with their entirely different types of chemical structures which make them foreign substances to cells and organisms, are, nevertheless, undergoing chemical changes in the organisms which have taken them up, changes that are caused by endogenous enzymes. While some foreign substances cause no effects whatsoever before they are eliminated in an unaltered form in urine, feces, or exhaled air, there are others that are biologically active by themselves. But, there are also those that become biologically active only as a result of the chemical changes to which they are subjected in the organism. The products of such transformations vary according to the chemical structure of the xenobiotic and the biochemical nature of the transformation and, for similar reasons, the new products may be biologically inert and nontoxic and easily eliminated, or they too may cause acute toxic damage in cells and organs or may have mutagenic or carcinogenic effects.

Environmental toxicology can, therefore, no longer limit its scientific research to risk limitation and risk assessment. Today we must determine the chemical reactivity of a xenobiotic in a biological system as well; we must delineate the metabolic pathways at an early stage of research, both qualitatively and quantitatively. We also must identify the various metabolites and assess their potential biological activities. Today, in other words, environmental toxicology will have to become biochemistry at its finest and that is why a biochemist feels quite at home at a conference like this one. However, this very thought can be developed even one

step further without taking the risk of becoming futuristic. That is to say, since man has elected to become dependent on modern and steadily advancing technology, environmental toxicology will be challenged to protect him from the by-products of his technologic advances. It will have to protect him not only by defining exposure limits and as a form of passive protection, but also by taking advantage of the nature of the chemical transformations to which xenobiotics are subject in biological systems and organisms. This form of active protection must be based on a complete and thorough understanding of the chemical structures and the biochemical, and even the immunologic, mechanisms and principles that are involved or that may have to be invoked in order to render such protection.

It is this recognition of present needs and requirements by a modern and responsive toxicology science that will cast the directions and format of its future research, and it is this recognition of the future which, I am happy to say, depicts itself in the promising program of this conference.

SESSION I

ANIMAL MODELING OF CARCINOGENESIS

Chairman

**Gary D. Stoner, Ph.D.
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**MECHANISMS OF ASBESTOS-INDUCED CARCINOGENESIS IN
HAMSTER TRACHEA**

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INTRODUCTION

In a recent survey of National Occupational Hazards⁽¹⁾, asbestos was ranked first on the list of carcinogens most hazardous to American industry. A linear dose-response relationship between the cumulative dosage of asbestos and the development of bronchogenic carcinoma has been reported in miners, millers, and factory workers^(2,3). Moreover, the demonstration of asbestos fibers in the lungs⁽⁴⁾ and urine⁽⁵⁾ of the general population has raised public concern regarding the possible health effects of environmental exposure to asbestos.

Epidemiologic studies have documented the important synergistic effect of smoking on the development of bronchogenic carcinoma in asbestos workers⁽⁶⁻⁸⁾. For example, persons occupationally exposed to asbestos (smokers and nonsmokers as a group) have an 8-fold higher incidence of tumors than the general population. Whereas there is little increase in disease (1.5-4 fold) among nonsmoking asbestos workers, smokers have an 80-92 fold higher predisposition.

A number of investigators have attempted to induce neoplasms in rodents after intratracheal instillation of asbestos or prolonged periods of exposure in inhalation chambers⁽⁹⁻¹⁸⁾. In general, the results of these experiments are difficult to interpret because of the small numbers of tumors and incomplete documentation of the types of malignancies observed. However, data show a striking increase in bronchogenic tumors when polycyclic aromatic hydrocarbons (PAH) (i.e. chemical carcinogens found in cigarette smoke) are administered at the same time as asbestos. These observations not only support the results of epidemiologic studies but also establish the critical role of PAH in tumor induction.

What is the mechanism of asbestos-induced carcinogenesis in the respiratory tract? Although asbestos has been termed a physical or "solid-state" carcinogen in the induction of mesothelioma, a rare type of malignancy of the lining cells of the body cavities, both epidemiologic and experimental evidence suggest that the pathogenesis of bronchogenic carcinoma is more complex. Because studies using administration of asbestos to laboratory animals have failed to provide insight into the cocarcinogenic effects of asbestos and cigarette smoke, we have addressed these problems using organ and cell cultures of hamster tracheobronchial epithelium^(19,20). A necessary extension of these studies was the development, using implantation of asbestos-exposed tracheal organ cultures into syngeneic animals, of a model for the induction of tumors^(21,22). Using both morphologic and biochemical approaches, we have examined the effects of asbestos, alone and with PAH adsorbed to the surface of the fiber, on the target cell of the respiratory tract.

METHODS

ASBESTOS

"Asbestos" is a generic name for a family of hydrated silicates having different chemical and physical features. Although we have examined comparatively the effects of crocidolite, amosite and chrysotile asbestos on the epithelium of the respiratory tract⁽²¹⁻²⁴⁾, the experiments described below were performed using crocidolite (UICC reference sample).

COATING OF THE PAH, 3-METHYLCHOLANTHRENE (3 MC) ON CROCIDOLITE

MC was adsorbed to heat-sterilized preparations of crocidolite asbestos using a modification of the technique of low temperature precipitation described by Saffiotti⁽²⁵⁾.

A solution of ¹⁴C-3MC, 2.9 or 0.29 mg (specific activity = 60.2 mCi/mmol, New England Nuclear, Boston, Mass.) in acetone was combined with unlabelled 3 MC (0.29 g, Aldrich Chemical Company, Milwaukee, Wisc.) at a ratio of 1:1,000. An equal weight of crocidolite (0.29 g) was added in cold acetone to the hydrocarbon, and the suspension agitated on a magnetic stirrer for 48 hours. At this time the 3 MC-coated asbestos was allowed to settle and the residual acetone was removed under nitrogen. The preparations then were washed 2x with distilled water to remove unbound 3 MC⁽²⁶⁾.

PREPARATION AND EXPOSURE OF TRACHEAL ORGAN CULTURES TO 3 MC-COATED ASBESTOS

The procedure for establishing tracheal organ cultures and their exposure to crocidolite is illustrated in Figure 1. Explants were prepared from the tracheas of 6- to 8-week-old female 'golden Syrian hamsters of the BIO 15.16 strain (TELACO, Bar Harbor, Me.) by previously described methods⁽¹⁹⁾. In comparison to randomly bred hamsters and other inbred strains, the BIO 15.16 hamster has demonstrated susceptibility to induction of carcinomas after administration of PAH⁽²⁷⁾. In brief, the trachea was excised, using sterile technique, from the larynx to the bronchial bifurcation and immersed in Hanks' balanced salt solution (HBSS) containing 100 µg/ml gentamicin and 25 U/ml mycostatin. After removal of extraneous tissue by dissection, the trachea was opened along the anatomical discontinuity in the cartilage rings. It was then cut into quarters and the segments grouped in 35 mm plastic Petri dishes that had been scored to allow adherence of the nonepithelial surface.

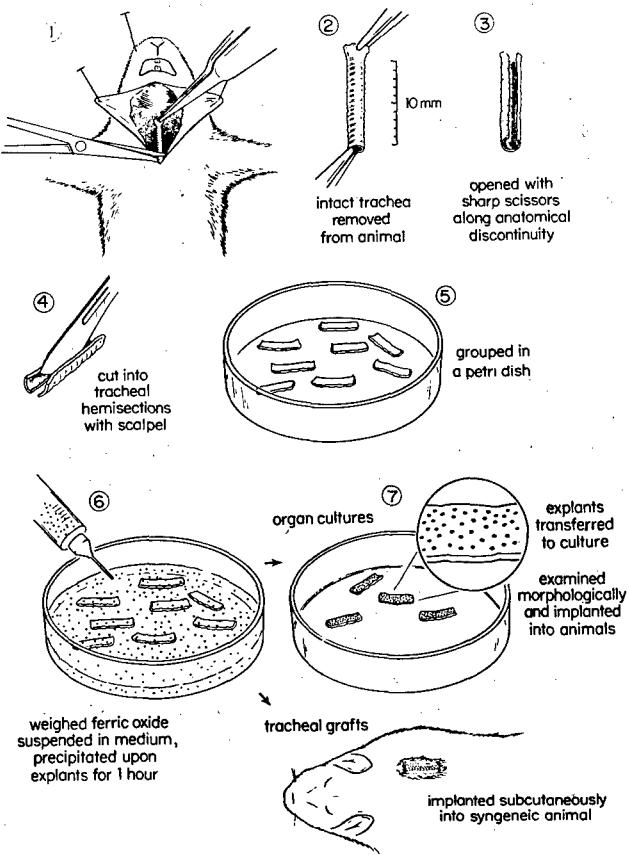


Figure 1. Schematic diagram of the procedures for establishing hamster tracheal organ cultures and their implantation into syngeneic animals.

Organ cultures were immersed for 1 hour in HBSS containing a suspension of either crocidolite or crocidolite with 3 MC. The explants then were transferred to dishes containing fresh medium and incubated at 35°C in a humidified 5% CO₂-95% air environment.

MORPHOLOGIC ASSESSMENT AND IMPLANTATION OF ORGAN CULTURES INTO HAMSTERS

After incubation for periods of time as long as 4 weeks, organ cultures were examined morphologically or implanted subcutaneously into syngeneic weanling female hamsters. For light microscopy, explants were fixed *in situ* using Bouin's solution; 5-μm paraffin sections then were prepared and stained with hematoxylin and eosin. For examination by transmission electron microscopy (TEM), tissues were fixed *in situ* in 4% glutaraldehyde postfixed in 1% osmium tetroxide, dehydrated, and embedded in Epon using standard methods. Sections (ca. 1 μ thickness) were prepared with an ultramicrotome and stained with toluidine blue. Ultra-thin sections were stained with uranyl acetate and lead citrate and examined with a Philips 300 microscope.

Specimens also were prepared for scanning electron microscopy (SEM) and x-ray energy spectrometry (XES) to localize the sites of asbestos deposition in the mucosa. After fixation in glutaraldehyde, tissues were critical point freeze-dried and coated with palladium in a vacuum evaporator. Explants were viewed with a JEOL JSM scanning electron microscope and analyzed by XES using a Kevex attachment.

In preliminary studies, organ cultures were implanted subcutaneously into syngeneic female weanling hamsters to assess their neoplastic potential. The epithelial surface of the tracheal segment was sutured along the midline at both ends to the underlying musculature of the animal. Hamsters were palpated for tumors at 3-week intervals and masses excised at 5 mm in diameter.

RESULTS AND DISCUSSION

Our work thusfar has addressed the important questions: 1) How does asbestos interact with the tracheal epithelial cell?; and 2) What biologic mechanisms account for the synergistic or (co)carcinogenic effects of asbestos and cigarette smoke?

INDUCTION OF TUMORS

Figure 2 summarizes the results of studies after implantation of asbestos-exposed tracheal grafts into syngeneic animals. Use of radiolabelled 3 MC allowed us to determine the amount of hydrocarbon on each tissue at the time of grafting. Tumors failed to appear in tissues treated with crocidolite alone. In contrast, the development of tumors was dosage dependent on the

amount of 3 MC adsorbed to the fibers. Eight of sixteen animals developed malignancies at highest concentrations of the hydrocarbon. Although the majority of the neoplasms were carcinomas, sarcomas, undifferentiated and mixed (i.e. carcinomas and sarcomas) tumors also were observed. The latency period of developing neoplasms ranged from 19 to 60 weeks.

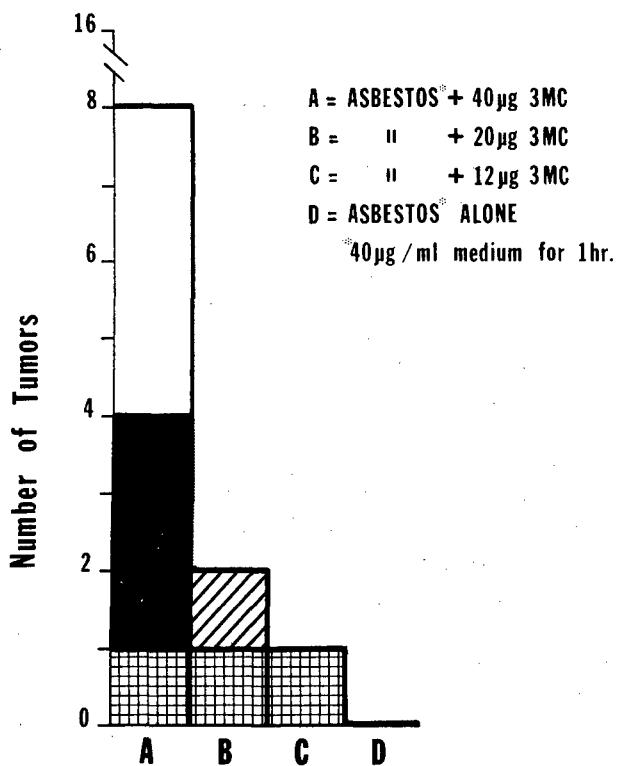


Figure 2. Dosage dependent induction of tumors using 3-Methylcholanthrene (3 MC) adsorbed to crocidolite asbestos. Poorly differentiated carcinomas (White solid); Poorly differentiated sarcomas (Dark solid); Well differentiated carcinomas (Diagonal bars); Undifferentiated malignancies (Cross-bars).

Our observations suggest that asbestos alone is non- or weakly carcinogenic in the respiratory epithelium. Since asbestos is not a potent mutagen⁽²⁸⁾ and inconsistently causes chromosomal aberrations in mammalian cell lines⁽²⁹⁻³¹⁾, its role as a complete carcinogen is improbable. Moreover, neither crocidolite nor chrysotile asbestos causes single-strand breakage of DNA in tracheal epithelial cells when examined by alkaline elution (Mossman, Eastman and Bresnick, manuscript in preparation).

ASBESTOS AS A PROMOTER OF CARCINOGENESIS

Carcinogenesis is thought to be a series of events that can be divided into sequential stages of initiation and promotion⁽³²⁾. An initiator interacts with the DNA of the target cell--the event resulting in malignant transformation. In contrast, a promoter generally is neither mutagenic nor carcinogenic, although its application is required for the development of a neoplasm.

Studies using a variety of tumor promoting substances, the most well-characterized being the family of phorbol esters, have defined certain parameters which appear intrinsic to their mechanism(s) of action. These include: a) attachment and/or entrance into target cells; b) stimulation of cellular division; c) inhibition of normal differentiation; and d) induction of ornithine decarboxylase (ODC), the rate-limiting enzyme in the biosynthesis of polyamines (reviewed in 33). These promoting events appear essential in the transformation of cells exposed to low dosages of chemical carcinogens such as PAH. Our work over the past several years has indicated that asbestos possesses many properties of a classical tumor promoter.

Using ultrastructural approaches, we have documented the phagocytosis of asbestos by tracheal epithelial cells and the association of primary lysosomes with phagosomes containing asbestos⁽³⁴⁾. Fibers appear to be transferred both intracellularly and intercellularly to the basal lamina and submucosa. One can envision, therefore, the asbestos fiber as a "carrier" of adsorbed PAH from cigarette smoke or the urban environment⁽³⁵⁾ into the target cell. Moreover, the rapid transfer of PAH from asbestos fibers to artificial cell membranes has been documented by others⁽³⁶⁾.

A well-established concept is the importance of cell damage and proliferation in the carcinogenic process⁽³⁷⁾. For example, Marquardt has shown that cells are transformed by chemical carcinogens *in vitro* only when undergoing DNA synthesis⁽³⁸⁾. In this regard, all types of asbestos, when compared to a variety of nonfibrous minerals, are cytotoxic to the tracheal epithelium although to varying degrees⁽²⁴⁾. If high concentrations of asbestos are added to organ cultures, superficial cells slough, whereas the basal layer remains intact and proliferates thereafter. After exposure to crocidolite and amosite asbestos, squamous metaplasia ensues, but can be reversed by addition of synthetic vitamin A analogs (i.e. retinoids) to culture medium⁽³⁹⁾. We have documented recently the induction of ODC activity during asbestos-induced proliferation⁽⁴⁰⁾.

SUMMARY

The information accrued thusfar from our studies is diagrammed schematically in Figure 3. In this hypothetical construct, crocidolite serves not only as a carrier of PAH into the tracheal epithelial cell, but also as a tumor promoter. For example, hyperplasia and dedifferentiation of the epithelium are observed. Our work with a variety of particulates in comparative studies^(41,42) indicates that asbestos is a "unique" mineral in the induction of squamous metaplasia. One might expect that the rapidly dividing basal cells in these lesions are more susceptible to transformation because of the accessibility of their DNA to the active metabolites of PAH.

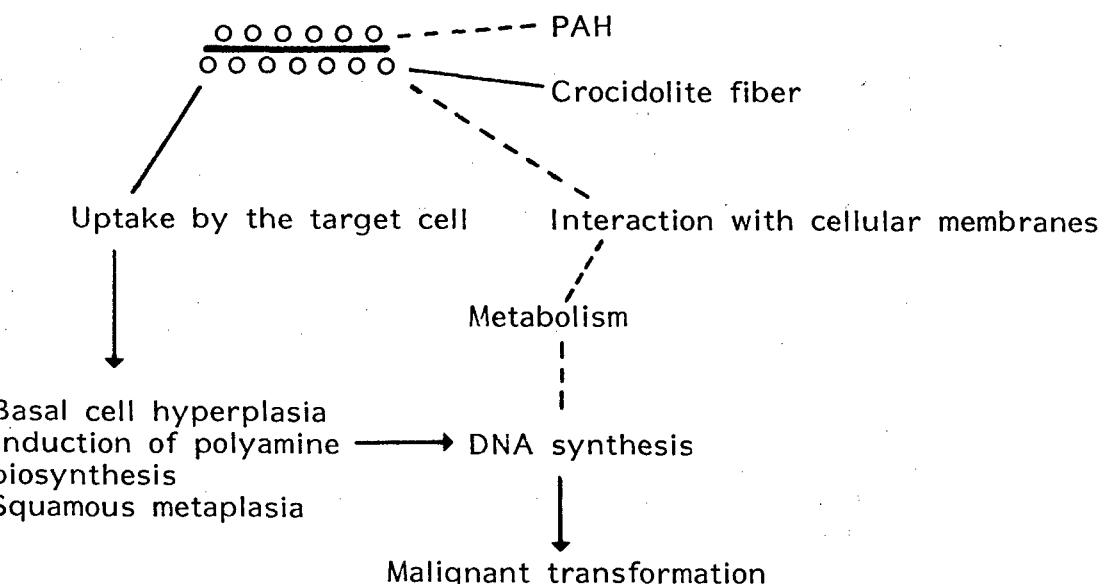


Figure 3. Hypothetical construct of the mechanism of asbestos-induced carcinogenesis in hamster trachea.

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CHEMICAL CARCINOGENESIS IN CULTURED RAT TRACHEAL EPITHELIUM

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INTRODUCTION

Respiratory tract epithelium is a critical site for cellular interaction with environmental contaminants. To understand the mechanisms of tumor induction in the target tissue, several experimental models have been developed (reviewed by Nettlesheim and Griesemer, 1978). The recent advances in the ability to culture respiratory tract epithelium have permitted the genesis of *in vitro* studies which promise to elucidate many cellular and biochemical processes in carcinogenesis which have been inaccessible in studies with whole animals.

Three *in vitro* model systems using rat trachea have been developed with the aim of studying respiratory tract carcinogenesis (Steele et al., 1977,79,80; Pai et al., 1982). We have chosen the direct acting carcinogen, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), and the polycyclic aromatic hydrocarbon, benzo(a)pyrene (B(a)P), which requires metabolic activation, as model compounds for these studies. In the first system tracheal epithelium is exposed to the carcinogen MNNG while in organ culture. This method is used to preserve tissue integrity and to maintain proliferation and differentiation similar to that found in the *in vivo* mucociliary epithelium. The epithelial cells are then allowed to grow off the explants in order to provide rapidly expanding primary cultures that can be maintained for at least 4 to 6 months *in vitro*. Cell lines can subsequently be established from such primaries and tested for oncogenicity. The second system utilizes a tracheal epithelial cell line which is derived from an explant following exposure to the tumor promoter 12-O-tetradecanoylphorbol-13-acetate, TPA (Steele et al., 1978). This cell line metabolizes B(a)P to metabolites qualitatively similar to those found in whole tracheas in organ culture (Cohen et al., 1979). In the third system, primary cultures of rat tracheal epithelium are exposed to MNNG; cell

lines are established from the primaries and some prove to be neoplastically transformed. The latter two systems offer far more quantitative and rapid systems to assess malignant transformation than the first.

METHODS

ORGAN CULTURE - CELL CULTURE SYSTEM

Tracheal explants are prepared from 10-to-12-week-old female Fischer 344 rat tracheas as previously described by Marchok et al., 1975. The explants (2 x 3 mm) are placed luminal side up on a 1.2 μ m Gelman TCM filter supported by a stainless steel grid in a conventional organ culture dish. The medium used for the organ culture phase consists of Waymouth's MB 752/1 plus 0.1 μ g insulin and 0.1 μ g hydrocortisone/ml and 2% fetal bovine serum. On days 3 and 6 the organ cultures are exposed to 0, 0.001, 1 or 10 μ g MNNG/ml in Waymouth's alone for 6 hours. Immediately following the last exposure the explants are placed on the bottom of tissue culture dishes to initiate epithelial outgrowths and establish primary cultures. The dishes contain an enriched medium (Waymouth's + 10 μ g insulin and 0.1 μ g hydrocortisone/ml + 10% fetal bovine serum + additional amino acids, sodium pyruvate, fatty acid, and putrescine) as described by Marchok et al. (1976). Re-planting the explants produces multiple outgrowths and primaries. If these cells could be dissociated and replated, after maintenance for a period of time in primary culture, and could be subcultured at least 5 times, they were designated "cell line cultures". Tumorigenicity of the cell line cultures during passage was determined by injecting 10^6 cells into the thighs of isogenic rats immunosuppressed by thymectomy and x-irradiation.

TRACHEAL CELL LINE SYSTEM

A nontumorigenic, pure epithelial cell line was established following exposure of an adult rat tracheal explant to TPA (Steele et al., 1978). At 24 hours after 4×10^5 epithelial cells were seeded onto 60 mm plastic dishes, the attached cells (1×10^5 /dish) were exposed for 24 hours to the following compounds: B(a)P, its proximal carcinogenic metabolite (\pm) r-7,t-8-dihydro-7,8-dihydroxy B(a)P (the 7,8-dihydrodiol), or (\pm) r-7,t-8-dihydroxy-t-9,10-oxy-7,8,9,10-tetrahydro B(a)P, also called diol epoxide I (the form suggested by many to be the major ultimate carcinogen from B(a)P). The cultures were grown to confluence and subcultured weekly. At the 10th passage (each passage represents approximately 3.3 population doublings) 10^6 viable cells from each subline were inoculated into immunosuppressed rats.

TRACHEAL PRIMARY CELL CULTURE SYSTEM

Freshly isolated tracheal epithelial cells were seeded (10^4 / 60 mm dish) onto a collagen substratum*. A modified F12 medium containing 3T3 fibroblast conditioned medium, insulin, hydrocortisone, transferrin, and bovine hypothalamus fraction was used to culture the cells. The primary cultures were exposed singly or up to eight times to 0.1 μ g MNNG/ml in serum-free medium. Patches or foci of epithelial cells remaining on the dishes at 18 or 30 days were counted by macroscopic observation. The cells were counted at Day 30 by dissociating the cultures and using a hemacytometer to count a known aliquot. Subculture was performed using 0.2% trypsin + 0.2% EDTA.

RESULTS

ORGAN CULTURE - CELL CULTURE SYSTEM

Primary cultures from both MNNG-exposed and control explants appeared morphologically similar during the first 3 months after carcinogen exposure. The earliest sign of carcinogen exposure was the appearance of focal areas of rapidly proliferating cells which were morphologically altered. We were able to establish cell lines only from primaries containing morphologically altered cells. Primary cell cultures from control explants did not produce foci of morphologically altered cells. The following effects of dose were observed: (1) higher doses yielded more long-term primary cell cultures per explant and more cell line cultures per explant; and (2) more explants exposed to higher doses produced tumorigenic cell lines and these cell lines produced tumors with shorter latency periods (Tables 1 and 2). We saw no significant differences between groups receiving different exposure concentrations in terms of the time to appearance of foci, or time to first subculture, or growth of a tumor once formed (Tables 2 and 3). The cell lines became increasingly malignant with time in vitro in terms of increased tumor incidence and shorter tumor latencies (Table 4). Adenosquamous cell carcinomas and keratinizing squamous cell carcinomas were obtained from cell lines in all groups (Table 5). Several tumorigenic cell lines produced tumors which metastasized to distant sites.

* Wu, R. (1981), Multiplication of normal rat tracheal epithelial cells in a hormone-supplemented medium, (submitted).

TABLE 1. MNNG EXPOSURE OF TRACHEAL EXPLANTS RESULTING IN THE ESTABLISHMENT OF ONCOGENIC AND NON-ONCOGENIC CELL LINES

MNNG Concentration (μ g/ml)	Number of Explants ^a Producing			
	Long-term Primary Epithelial Cell Cultures ^b	Cell Line Cultures	Tumorigenic Cell Lines By the 10th Passage ^d	Tumorigenic Cell Lines By the 20th Passage
0	6(13)	0	0	0
0.001	9(15)	7(8)	4(4)	5(6)
1.0	10(33)	8(13)	5(6)	6(9)
10.0	10(46)	10(20)	9(15)	9(15)

^a There were 10 explants in each group.

^b Epithelial cell cultures which survived 3 weeks or more after removal of the explant. The numbers in parenthesis indicate the total number established in each group. Since each explant was replated 10 times, the maximum possible number is 100.

^c Cell line cultures can be repeatably subcultured.

^d Cell lines were inoculated i.m. and inoculation sites were observed a maximum of 300 days. Most tumors appeared by 100 days although some appeared as late as 280 days after inoculation.

(Data from Steele et al., 1979)

TABLE 2. COMPARISON OF TIME INTERVALS MEASURED IN THE TUMORIGENESIS ASSAY WITH CARCINOGEN EXPOSURE CONCENTRATION

MNNG Concentration (μ g/ml)	Number of Explants or Cell Lines ^a	Mean Passage Number	Mean No. Days From				
			Exposure to Inoculation Which Produced A Tumor	Exposure to Appearance of Tumor	Establishment of a Cell Line to Appearance of Tumor	Inoculation to Tumor Appearance	Tumor Appearance to Sacrifice at 3.0 cm Diameter
0.001	5	12	323(± 9) ^b	453(± 35)	153(± 8)	108(± 13) ^c	74(± 20)
1.0	6	14	317(± 21)	414(± 34)	128(± 19)	63(± 13)	60(± 9)
10.0	9	12	322(± 14)	392(± 26)	115(± 22)	56(± 21) ^c	86(± 9)

^a In cases where explants yielded two or more cell lines, only that cell line which showed evidence of neoplastic transformation first was considered.

^b Days (\pm S.E.)

^c Significantly different at $p < 0.05$.

(Data from Steele et al., 1979)

TABLE 3. THE ESTABLISHMENT OF EPITHELIAL CELL LINES FOLLOWING EXPOSURE TO VARIOUS CARCINOGEN CONCENTRATIONS

MNNG Concentration (μg/ml)	Number of Cell Lines Established	Appearance of Rapidly Proliferating Foci ^a	Appearance of Morphologically Altered Cells	First Subculture ^c	Fifth Subculture ^d
0.001	8	147 (\pm 13)	168 (\pm 8)	202 (\pm 20)	276 (\pm 10)
1.0	13	123 (\pm 13)	166 (\pm 11)	190 (\pm 11)	275 (\pm 15)
10.0	20	135 (\pm 9)	160 (\pm 7)	197 (\pm 12)	269 (\pm 11)

^a Time at which focal areas of primary cell cultures appeared to be rapidly proliferating as evidenced by numerous mitotic figures and a multilayering of cells.

^b Time at which cells were observed that had a high nuclear-to-cytoplasmic ratio and grew rapidly across the surface of the dish.

^c Time of first successful dissociation, usually after 10^6 of the morphologically altered cells had accumulated in the dish.

^d Populations of tracheal epithelial cells which can be subcultured 5 times from permanently proliferating cell lines. Cells were subcultured when they reached confluence.

(Data from Steele et al., 1979)

TABLE 4. TIME IN VITRO AND PASSAGE NUMBER COMPARED TO IN VIVO TUMORIGENICITY FOR EPITHELIAL CELL LINES INOCULATED AT DIFFERENT TIMES

Cell Line Designation	Days From Exposure to Inoculation (In Vitro Time)	Passage Number	Tumor Incidence ^a	Mean No. Days From Inoculation to Tumor Appearance (In Vivo Time)
5-10-3	346	11	4/4	81
	402	20	4/4	60
2-10-10	212	5	1/4	160
	250	10	3/4	135
7-10-6	250	9	1/4	192
	327	20	2/4	164
7-10-9	346	15	2/4	122
	409	25	4/4	53
8-1-1	253	10	2R/4 ^b	--
	358	20	3/4	68
	389	25	2/2	53
4-1-2	242	7	4R/4 ^c	--
	285	12	4/4	127

^a Two immunosuppressed animals were inoculated in each thigh with 10^6 viable cells. The animals were palpated weekly for 300 days for tumor development.

^b Two injection sites formed nodules which regressed (R). No epithelial cells were found upon microscopic examination at the injection site at 300 days.

^c Initially formed small tumors that regressed. A very small clump of cells were detected microscopically at two of the inoculation sites when sacrificed 300 days after inoculation.

(Data from Steele et al., 1979)

TABLE 5. HISTOLOGIC CLASSIFICATION OF TUMORS DERIVED FROM MNNG-INDUCED CELL LINES

Concentration ($\mu\text{g/ml}$)	Number of Tumorigenic Cell Lines	No. of Tumorigenic Cell Lines Which Produce Metastasis	Histologic Type of Carcinomas		
			Adeno Squamous	Poorly Differentiated Squamous	Squamous
0.001	6	0	2	0	4
1	9	2	5	1	3
10	15	4	9	1	5
TOTAL	30	6	16	2	12

(Data from Steele et al., 1979)

TRACHEAL CELL LINE SYSTEM

Our previous studies show that primary cell cultures of tracheal epithelium and the nontumorigenic tracheal epithelial cell line, 2Cl, possess the necessary enzymes for the metabolic activation of the polyaromatic hydrocarbon, benzo(a)pyrene (Cohen et al., 1979). This cell line therefore appeared to be suitable to study epithelial transformation, since we could perform many more *in vitro* measurements on a rapidly growing cell line than with a slowly growing primary cell culture. To determine exposure concentrations, we estimated the relative toxicity of B(a)P and its metabolites using a decrease in colony forming efficiency (CFE) as a measure of toxicity. Two exposure concentrations for each compound were then chosen for the *in vitro* transformation attempt: a "nontoxic" concentration (i.e. little or no decrease in CFE compared to controls) and a "toxic" concentration (i.e. a 50% decrease in CFE compared to controls). The CFE of the cells exposed to B(a)P varied between 70-90% of control values, even up to concentrations at the solubility limit of the medium (40 μM), while the 7,8-dihydrodiol B(a)P and the 9-10-epoxide B(a)P were toxic at much lower concentrations.

Nearly confluent cultures of 2Cl cells were exposed 24 hours to B(a)P, 7-8 diol BP and diol epoxide B(a)P at "toxic" and "non-toxic" concentrations as shown in Table 6. All exposed sublines and controls were inoculated into immunosuppressed isogenic animals after 10 passages. After a 100 day observation period, only one of the eight sublines exposed to B(a)P formed tumors, while one half and all of the 7,8 diol B(a)P and diol epoxide B(a)P exposed sublines, respectively, formed tumors. Tumors from the sublines have been histologically examined and were differentiated squamous and adenosquamous cell carcinomas. The suggested ultimate carcinogen, diol epoxide-B(a)P, was 100 percent efficient in transforming the 2Cl cell line even though the molar concentration was

1/100th that of the B(a)P. It is also noteworthy that "nontoxic" concentrations of the compounds tested are still able to transform cells, since this will greatly simplify the interpretation of future *in vitro* transformation data.

TABLE 6. TUMORIGENICITY OF THE 2C1 TRACHEAL EPITHELIAL CELL LINE FOLLOWING A SINGLE 24 HOUR EXPOSURE TO BENZO(A)PYRENE, 7,8 DIHYDRO-7,8-DIHYDROXY BP, AND 7,8 DIHYDRO-9,10 EPOXIDE BP

<u>Compound</u>	<u>Exposure Concentration^a</u>	<u>No. Independent Sublines</u>	<u>No. of Tumorigenic Sublines After 10 Passages^b</u>
Control	0	4	0
B(a)P	8 μ M	4	2
	40 μ M	4	0
7,8 dihydro-7,8-dihydroxy BP	4 μ M	4	2
	20 μ M	4	3
7,8-dihydro-9,10 epoxide BP	0.08 μ M	4	4
	0.4 μ M	4	4

^a DMSO concentration in all media was 0.2%.

^b Tumorigenicity was determined by inoculating 10^6 viable cells i.m. into the thighs of 2 immunosuppressed isogenic hosts. The inoculation site was palpated weekly over the 100 day observation period.

(Data from Steele et al., 1980)

TRACHEAL PRIMARY CELL CULTURE SYSTEM

Transformation studies of epithelial cell lines, while easier and more rapid than explant outgrowth studies, do not allow analysis of early events in normal cells following carcinogen exposure. To this end we have developed a primary tracheal transformation system. Quantitative measurements of cell number, number and density of atypical foci, and frequency of cultures yielding established epithelial cell lines were analyzed as a function of carcinogen dose (Table 7). By day 18 a marked difference could be seen between carcinogen exposed cultures and control cultures. Carcinogen exposed cultures contained proliferating compact epithelial cells, strikingly different from the nonproliferating large flattened cells in the control cultures. The numbers of

these "atypical foci" increased with increasing numbers of exposures. By estimating the cell density and size of these foci at Day 18, we could indirectly determine the cell number of the entire dish. We found that MNNG exposed cultures, in general, contained 15 to 100 times as many cells as control cultures. At Day 30 the numbers increased approximately 10 fold in carcinogen treated cultures due to the expansion of the atypical foci.

TABLE 7. PROGRESSION OF PRIMARY EPITHELIAL CELL CULTURES AND CELL LINES TO NEOPLASTIC TRANSFORMATION FOLLOWING MNNG EXPOSURE

No. of MNNG Exposures to 0.1 μ g/ml	Mean No. of Epithelial Patches at Day 18 ^a	Mean No. of Epithelial Cells		No. of Cell Lines Established by Day 90 per Cultures Attempted ^d	No. Anchorage Independent Cell Lines per Cell Lines Tested ^e
		Day 18 ^b	Day 30 ^c x10 ⁻⁵		
0	0.4	0.04	0.02	0/30	0/0
1	2.3	0.61	4.59	2/15	0/1
2	5.6	2.21	9.76	4/9	2/2
4	6.0	5.29	18.23	6/12	1/3
6	4.6	6.42	18.01	7/10	6/8
8	10.8	4.84	22.36	4/6	3/3

^a Scored by macroscopic observation.

^b Indirect count estimates.

^c Direct cell counts by hemacytometer.

^d The first subculture was attempted on Day 30. Cell lines status infers that 5 successful subcultures were achieved in 60 days.

^e In each case 10⁵ cells were tested, cultures having 10 or more colonies were considered positive.

(Data from Pai et al., manuscript submitted, 1982)

At Day 30 all cultures were dissociated and subculture was attempted. If cultures survived 5 passages they were designated "cell lines". Cultures which received one DMSO exposure could not be subcultured and no cell lines could be established of the 30 control primaries tested so far. In contrast, 13 to 70% of the MNNG exposed cultures achieved the cell line status.

The cell lines have been tested for anchorage independent growth at the twentieth passage in 0.3% agarose. In 70% of the cases the epithelial cells had achieved anchorage independence, suggesting that neoplastic transformation had either occurred or was imminent (see Terzaghi and Nettesheim, 1979; Marchok et al., 1978).

DISCUSSION

The culture systems described here represent three models for in vitro malignant transformation studies of respiratory epithelial tissue. The various advantages and disadvantages of these epithelial systems are discussed below.

The organ culture system has several unique characteristics which mimic the in vitro situation. The carcinogen exposure was carried out with tracheal explants which have a well-differentiated, slowly proliferating mucociliary epithelium. The normal epithelial cell architecture and epithelial-mesenchymal interactions are maintained in the organ culture state. Therefore, the tissues during exposure closely resemble the tracheal mucosa in vivo. However, it is difficult to assess carcinogen induced cellular toxicity or perform biochemical analysis on the epithelial cells of this complex tissue.

The cell line system offers several advantages compared to the organ culture system. First, known numbers of attached cells which will form colonies are exposed to the carcinogen; thus, equitoxic exposures can be performed. Secondly, the time required for malignant transformation to be expressed was only 10 weeks compared to 6 to 8 months for the organ culture system. Thirdly, only epithelial cells are exposed so that indirect effects of other tissue types are eliminated and direct biochemical measurements can be made on the epithelial cells. The main disadvantage, however, in working with a cell line is that one or more of the steps of neoplastic transformation may already have occurred and one cannot study the entire process in cells which may be partially transformed.

The primary cell culture system has most of the advantages of the cell line system, but the primary system permits the study of effects of toxic agents on normal cells which are capable of normal differentiation, and senescing. These latter two aspects of normal cells appear to be key features in the initial alterations brought about by carcinogen exposure. Carcinogen exposure, such as those seen in our experiments and documented in other systems (see review by Franks and Wigley, 1979), induces cells to differentiate abnormally and escape senescence. Early preneoplastic foci such as those seen in our studies* may be extremely useful in themselves as endpoints for a short-term assay. They should also prove to be a "stepping stone" to discover other ultrastructural or biochemical markers of transformation.

* Pai, S. B., V. E. Steele, and P. Nettesheim (1982), Quantitation of early cellular events during neoplastic transformation of tracheal epithelial cell cultures, (submitted).

These epithelial cell culture systems are currently providing means for detailed studies of various phenotypic changes which occur in cells of the respiratory tract during oncogenic transformation.

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A MOUSE LUNG CARCINOMA MODEL SYSTEM*

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INTRODUCTION

Lung cancers have been induced in such diverse species as mice^(6,9,12,20), rats^(1,17,18), hamsters^(2,4,15,16), rabbits⁽⁵⁾ and dogs⁽¹⁴⁾ following intratracheal administration of polycyclic aromatic hydrocarbons. The types of tumors induced have included squamous cell carcinomas, alveolar adenocarcinomas, adenosquamous carcinomas, and poorly differentiated carcinomas. The location of such tumors extended from the larynx to the trachea, the main bronchi, the terminal bronchioles and the alveoli.

The mouse presents several unique characteristics which make it a useful model to study the mechanism(s) for the induction of lung cancer. The advantages of using the mouse for such studies are: a) availability of a large number of genetically diverse inbred strains; b) economy of operation; c) availability of colonies which are well defined in terms of their biological adventitious agents; and d) availability of strains of mice in which susceptibility to lung cancer is genetically regulated^(9,10). To further enhance the utility of the mouse system for carcinogenesis studies, more information is required on the type, location, biologic behavior and the degree of spontaneous occurrence of lung carcinomas. To this end, experiments were initiated in two strains of mice to determine the occurrence of spontaneous and chemically-induced lung tumors.

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MATERIALS AND METHODS

ANIMALS

BC3F1/Cum (C57Bl/Cum x C3H/Anf Cum) female and C3H/Anf Cum female mice were purchased from Cumberland View Farms (Clinton, TN) at 4-6 weeks of age and housed according to previously published procedures^(3,9). Mice were found to be serologically free of Reovirus type 3, pneumonia virus of mice, K virus, encephalomyelitis virus, polyoma virus, Sendai virus, minute virus of mice, mouse adenovirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, and ectromelia virus. Except for the study comparing the tumorigenicity of various chemicals, all mice were vaccinated with 0.1 ml Sendai vaccine before any chemical treatment.

CHEMICAL TREATMENT

3-Methylcholanthrene (MCA) and benzo(a)pyrene (BP) (Eastman Organic Chemicals, Rochester, NY) were recrystallized from benzene. (\pm)Trans-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene (BP-7,8-diol) was a generous gift from Dr. D. M. Jerina (NIAMD, NIH). These chemicals were ground lightly for approximately 20 minutes using a mortar and pestle and were suspended in solution of 0.2% gelatin in sterile saline (gel-saline) at a concentration of 1.0 μ Mole per 0.02 ml (~250 μ g). The suspension was stored at 4-6°C in aliquots sufficient for one day's use. The concentrations were determined fluorometrically before use.

Mice were 8-12 weeks old when first treated. The mice were lightly anesthetized with Metofane® (Pitman-Moore, Inc., Washington Crossing, NJ) and administered chemicals in 0.02 ml gel-saline or 0.02 ml gel-saline alone via intratracheal instillation according to previously published procedures^(9,10). Animals were treated every other week until the desired dose was given.

NECROPSY

Mice were observed twice daily for evidence of illness or respiratory distress. Dates and circumstances of death were recorded for all mice. Non-autolyzed tissues from mice found dead and those killed when moribund were examined microscopically. Lungs were fixed with approximately 1.5 ml of 10% buffered formalin by infusion via the trachea. The lungs were ligated at the trachea and the thoracic viscera removed as a single unit. Lung, trachea, esophagus and thoracic lymph nodes were sectioned (6 μ m) as a unit at 3 levels, using a frontal plane of section. Salivary gland, lymph nodes (cervical, bronchial), spleen, liver, kidneys, adrenal glands, large and small intestines, stomach, uterus, ovary, urinary

bladder, heart, thymus and head were also sectioned in 238 BC3F1/Cum mice. All tissues were stained with hematoxylin and eosin and examined microscopically.

MORPHOLOGIC CRITERIA

A brief description of the lung tumors observed in these studies is presented below. A detailed description will be presented elsewhere¹.

Squamous cell carcinomas (SCC) were nodular masses usually located in the peripheral portions of the lung. The masses were generally white to slightly yellow, often with red depressed centers. These masses were normally well-vascularized, firm, and smooth with irregular margins. SCC were composed of squamous epithelial cells which produced varying amounts of keratin. Vascular invasion was a prominent feature. The tumors appeared to arise from the alveoli or terminal bronchioles. Metastases usually occurred in the heart, kidneys, and bronchial lymph nodes.

Alveolar adenocarcinomas (AAC) occurred as discrete greyish-white, firm masses, located in the peripheral portions of the lung, and were often multiple⁽¹⁹⁾. Some tumors occasionally showed pleural invasion and metastasis to tracheobronchial lymph nodes. Adenomas were not included in this category.

Adenosquamous carcinoma (ASC) contained elements of both tumors described above. Whether these tumors reflect a "collision" of two different tumor types or represent a differentiation from one cell type to another was difficult to determine. Usually one component (squamous or glandular) was found in metastases.

A small number of other tumor types were observed in these studies. These will be described in detail elsewhere¹. These tumors were poorly differentiated carcinomas (PDC) and adenocarcinomas unclassified (AU).

The most likely causes of death from these various tumors are: a) lung infarctions; b) renal infarctions; c) congestive heart failure caused by obstruction of the left atrium; and d) anoxia as the result of pulmonary insufficiency.

¹ Pathology and Biologic Behavior of Chemically Induced Lung Tumors in Inbred Strains of Mice. L. H. Billups, C. J. Henry, B. Sass, M. D. Avery, L. A. Breth, W. C. Hall, R. E. Kouri, 1981, in preparation.

DATA ANALYSES

Survival data are given as mean life-span or mean survival time, defined as the sum of the number of weeks each animal lived divided by the total number of animals. Tumor data are presented as the probability of an animal dying with lung cancer after a specific period of time following chemical treatment^(8,11,16).

RESULTS

SURVIVAL

The mean life-span of untreated, shelf control BC3F1/Cum female mice was approximately 114 weeks, with a maximum life span of approximately 144 weeks. Survival of both BC3F1/Cum and C3H/Anf Cum mice following the vehicle treatment period was high and similar to the untreated, shelf control mice. Mean survival time post treatment for BC3F1/Cum mice was approximately 102 weeks and for C3H/Anf Cum mice was approximately 89 weeks. These correspond to approximately 114 and 101 weeks of age, respectively.

Survival times for MCA treated mice were dependent on dose and were much shorter than vehicle or shelf control mice. Mean survival time post treatment for BC3F1/Cum mice treated nine times with MCA was approximately 36 weeks on test. Mean survival times post treatment for C3H/Anf Cum mice treated 3x (750 µg), 6x (1500 µg), or 9x (2250 µg) with MCA were approximately 54 weeks, 46 weeks, and 40 weeks on test, respectively.

HISTOPATHOLOGY OF CONTROL MICE

Observations from control BC3F1/Cum and C3H/Anf Cum mice from the MCA study are presented in Tables 1 and 2, respectively. No differences were observed between shelf control and vehicle control BC3F1/Cum mice and the data from these groups were pooled (Table 1). No pulmonary tumors were observed in any control mice before 75 weeks. A total of 10 pulmonary AAC were observed in 140 BC3F1/Cum mice over the 138 week observation period (Table 1). The average expression time for these tumors was 105 weeks. One pulmonary AAC was observed at 91 weeks in 33 C3H/Anf Cum mice (Table 2). No evidence of pulmonary SCC was found in any of these control animals.

HISTOPATHOLOGY OF MCA TREATED MICE

BC3F1/Cum and C3H/Anf Cum mice that died following MCA treatment had a variety of pulmonary carcinomas. Over 95% of lung tumors observed were SCC, AAC, PDC and ASC. Table 3 presents a comparison of the distribution of pulmonary carcinomas as a function of time post MCA treatment of BC3F1/Cum mice. Three animals that died before the end of the treatment period were found to have SCC.

TABLE 1. HISTOLOGIC OBSERVATIONS FOUND IN
CONTROL BC3F1/CUM MICE^a

<u>Number of Animals</u>	<u>Diagnosis</u>	<u>Weeks on Test</u>	
		<u>Range</u>	<u>Mean</u>
41 (29%) ^b	Lymphosarcomas, reticulum cell sarcomas, and leukemias	47-131	104
10 (7%)	Fibrosarcomas	91-128	106
10 (7%)	Lung carcinomas	75-128	105
10 (7%)	Neoplasms of the mammary gland, sebaceous gland, Harderian gland, ovary, and of undetermined origin	73-133	106
4 (3%)	Hepatocellular carcinomas	99-125	111
9 (6%)	Adenomas of the lung ^c and liver	71-127	109
19 (14%)	Nephritis	93-138	109
9 (6%)	Pneumonia, congestion and lung inflammation	68-134	109
7 (5%)	Spleen and liver necrosis	89-114	102
21 (15%)	Died without observing a major disease, but such incidental findings were observed as myocarditis, atrial thrombosis, otitis media, otitis externa, uterine hydrometra, uterine hyperplasia, extramedullary hematopoiesis, uterine and ovarian cysts, chronic cystitis and hematocysts	28-131	91

^a Mice were necropsied when found dead or killed when moribund. Data are presented for the major findings which could have been the cause of death. A total of 140 BC3F1/Cum mice were evaluated.

^b Percent incidence.

^c Adenomas were benign alveologenic tumors, which lacked morphologic evidence of malignancy.

TABLE 2. HISTOLOGIC OBSERVATIONS FOUND IN
CONTROL C3H/ANF CUM MICE^a

Number of Animals	Diagnosis	Weeks on Test	
		Range	Mean
8 (24%) ^b	Carcinomas of the mammary gland	16-103	89
4 (12%)	Fibrosarcomas	78-121	95
4 (12%)	Hepatocellular carcinomas	87- 97	94
3 (9%)	Sarcomas of the reticuloendothe- lium, uterus, and of undetermined origin	46-103	74
1 (3%)	Lung carcinomas (AAC)	91	--
6 (18%)	Adenomas of the liver, mammary gland and pituitary gland	76-107	91
2 (6%)	Lung congestion and bronchitis	23- 34	29
5 (15%)	Died without observing a major disease, but such incidental findings were observed as myo- carditis, uterine hydrometra, ovarian cysts, otitis media, otitis externa and nematodiasis	55-100	94

^a Mice were necropsied when found dead or killed when moribund. Data are presented for the major findings which could have been the cause of death. A total of 33 C3H/Anf Cum mice were evaluated.

^b Percent incidence.

The first SCC was observed at 12 weeks after initiation of MCA treatment, after only five intratracheal MCA instillations. Of the 224 animals that died during 17-40 weeks on test, 190 (85%) were observed to have SCC alone or in combination with other malignant tumors. During the same time interval, only 49 (22%) of the animals were observed to have any evidence of AAC. However, of the 74 animals dying after 50 weeks on test, 67 (91%) expressed AAC alone or in combination with other tumors, and 27 (36%) expressed evidence of SCC.

Analysis of the biologic behavior of the MCA-induced SCC and AAC in 234 BC3F1/Cum mice showed that of the 172 SCC's observed, 90 (52%) were extensively invasive and/or metastasized to virtually all major organs, especially the heart, kidney, and bronchial lymph nodes. The most predominant route of metastasis was by direct invasion of the pulmonary vein with extension to the left atrium of the heart.

TABLE 3. DISTRIBUTION OF LUNG CANCERS IN BC3F1/CUM MICE AS A FUNCTION OF TIME AFTER 9 MCA TREATMENTS^a

Weeks on Test	Total ^c	No. Tumors	NUMBER OF ANIMALS ^b					
			SCC	AAC	Both SCC+AAC	SCC+ Other ^d	AAC+ Other ^e	Other Tumors ^f
0-16 ^g	---	-	--	--	---	-	-	-
17-19	2	2	0	0	0	0	0	0
20-29	80	8	66	1	1	2	0	2
30-39	142	6	87	12	33 ^h	1	2	1
40-49	76	0	29	9	31 ⁱ	2	2	3
50-59	54	1	4	26	19 ^j	0	4	0
60-69	13	0	0	8	2	0	1	2
70-79	4	0	0	3	1	0	0	0
80-89	2	0	0	1	1	0	0	0
90-99	1	0	0	1	0	0	0	0

^a Mice were treated with 9 intratracheal doses of 250 µg MCA in 0.02 ml GS at biweekly intervals for a total dose of 2250 µg MCA.

^b The diagnoses for 374 animals were individually arranged in 7 categories.

^c The number of animals that died or were killed when moribund is given for each time interval.

^d Includes 1 animal with SCC and lymphosarcoma (21 weeks), 3 with SCC and PDC (28, 43, and 48 weeks), and 1 with SCC and PDC (33 weeks).

^e Includes 1 with AAC and carcinoma of bronchogenic origin (37 weeks), 1 with AAC and AU (45 weeks), 4 with AAC and PDC (30, 46, 58 and 60 weeks), and 3 animals with AAC and ASC (51, 52, and 56 weeks).

^f Includes 5 animals with ASC (2 at 27 weeks, 2 at 40 weeks, 1 at 66 weeks), 1 with sarcoma (37 weeks), and 1 with AU and PDC (45 weeks), and 1 with PDC (62 weeks).

^g Animals (26 mice) that died during the treatment period are not included in these analyses. Three animals that died during this period had SCC (12, 15, and 16 weeks).

^h Includes 1 animal with SCC, AAC and PDC (34 weeks) and 1 with SCC, AAC, and fibrosarcoma (36 weeks).

ⁱ Includes 1 animal with SCC, AAC, and AU (43 weeks) and 1 with SCC, AAC, and PDC (48 weeks).

^j Includes 1 animal with SCC, AAC, and AU (53 weeks) and 1 with SCC, AAC, and sarcoma (57 weeks).

A total of 62 animals developed AAC alone or with malignancy other than SCC, while 70 had both SCC and AAC. Of the 62 animals with AAC, 29 (47%) showed extensive evidence of invasion and/or metastasis to various organs. AAC most often invaded the pleura, mediastinum and thoracic wall.

The distribution of lung cancers in C3H/Anf Cum mice as a function of dose of MCA is shown in Figure 1. The animals that died of tumors early after MCA treatment (less than 40 weeks) were usually found with SCC (overall mean of 85% for all of these

groups, Figure 1). Animals that died of late tumors after MCA treatment (greater than 50 weeks) were usually found with AAC (overall mean of 93% for all three groups, Figure 1). Different doses of MCA failed to alter this response. For all three MCA treated groups, the mean expression time for SCC was 33 ± 4 weeks, and for AAC it was 57 ± 6 weeks. As the dose of MCA was increased, the number of animals dying with evidence of SCC over the entire observation period was 19/72 (26%), 20/62 (32%), and 61/104 (59%) for the 3x (750 μ g), 6x (1500 μ g), and 9x (2250 μ g) MCA treatment groups, respectively. Conversely, as the dose of MCA increased, the number of animals dying with AAC decreased (46/72 (64%), 37/62 (60%), 55/104 (53%) for 3x, 6x, and 9x, respectively).

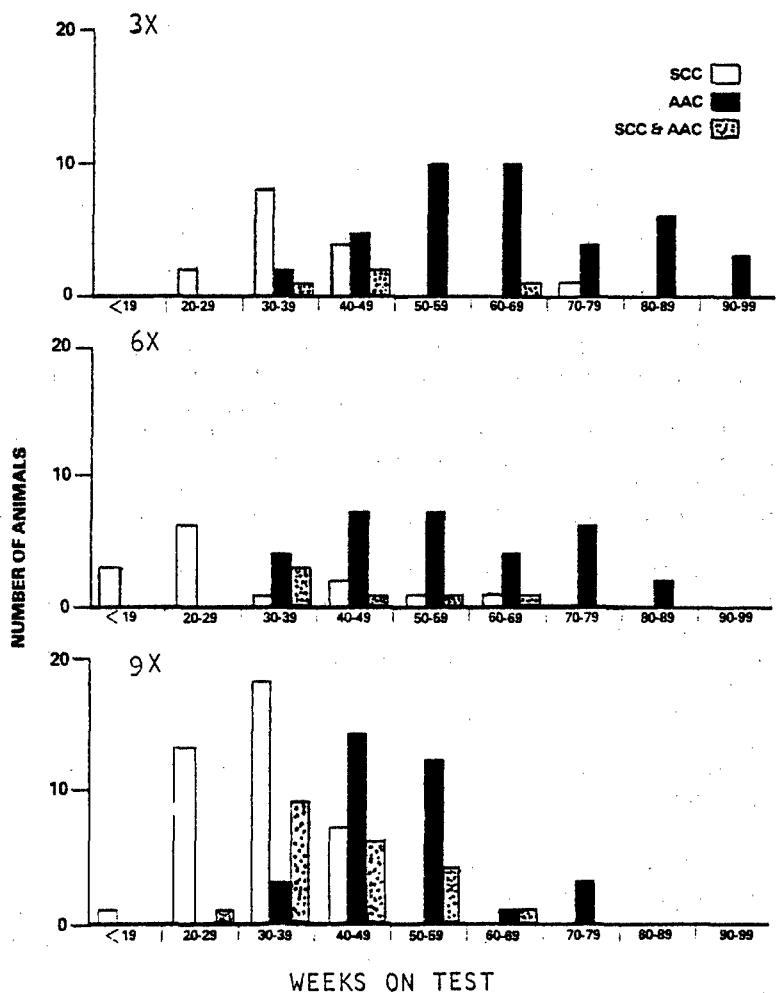


Figure 1. Distribution of lung cancers in C3H/Anf Cum mice as a function of time after 3, 6, or 9 doses of 250 μ g MCA. There were a total of 72, 62, and 104 mice in mice treated with 3, 6, or 9 doses of MCA, respectively.

PROBABILITY ANALYSIS

The data can be analyzed for the probability of mice dying with a lung carcinoma at any given time following carcinogen treatment. Figure 2 compares the cumulative probability of dying with lung cancer following treatment with 2250 μ g MCA in both BC3F1/Cum and C3H/Anf Cum mice. The probability of either BC3F1/Cum or C3H/Anf Cum mice dying of a lung tumor was identical over any given time interval. By 88 weeks after MCA treatment, the treated animals reached a cumulative probability of 0.99, whereas the probability of a control animal dying of a lung tumor was less than 0.006.

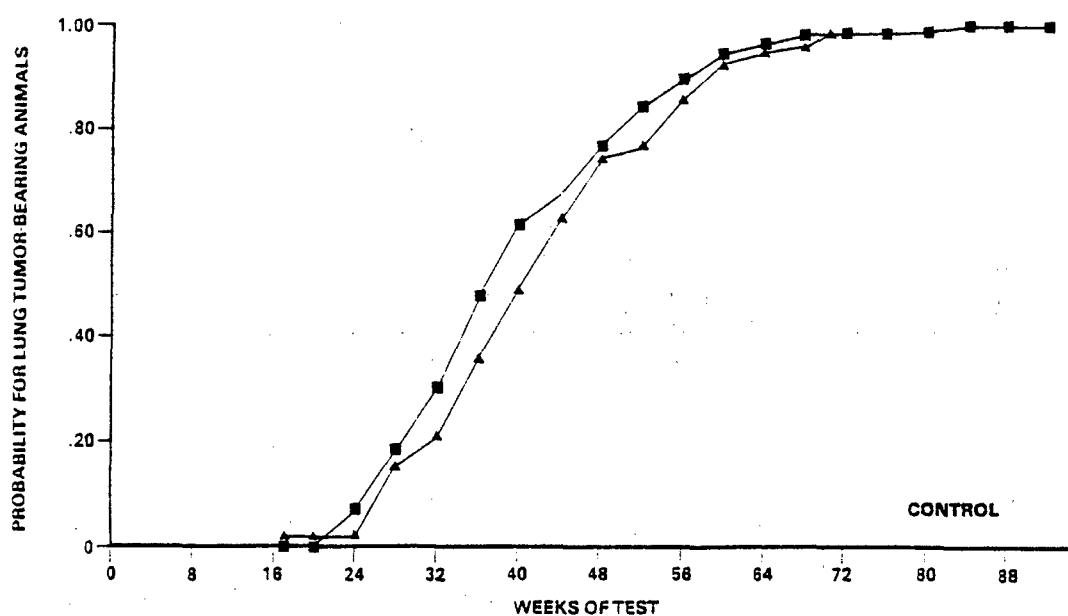


Figure 2. Probabilities for an animal dying of a lung tumor in BC3F1/Cum mice (▲) and C3H/Anf Cum mice (■) treated with 9 doses of MCA (2250 μ g MCA total). Data are calculated from 374 BC3F1/Cum mice and 113 C3H/Anf Cum mice. Over 95% of the BC3F1/Cum and 87% of C3H/Anf Cum mice died of lung cancer in these treatment groups.

Use of this method of analysis in C3H/Anf Cum mice treated with either 3 (750 μ g), 6 (1500 μ g), or 9 (2250 μ g) doses of MCA is presented in Figure 3. The probability of an animal dying of a lung tumor at any given time interval is related to the dose of MCA. Analysis of these data showed that 9 doses of MCA resulted in a significantly higher lung tumor probability than either 6 or 3 doses of MCA ($p < 0.001$). Animals treated with 6 doses had only a slightly higher lung tumor probability than animals treated with 3 doses ($p = 0.078$). By only 28 weeks after treatment, the probability of lung cancer was significantly higher ($p < 0.05$) in the animals treated 9 times compared with those treated 3 times. By 38

weeks after treatment, animals treated with MCA 9 times were significantly higher in tumor probability than those animals treated 6 times.

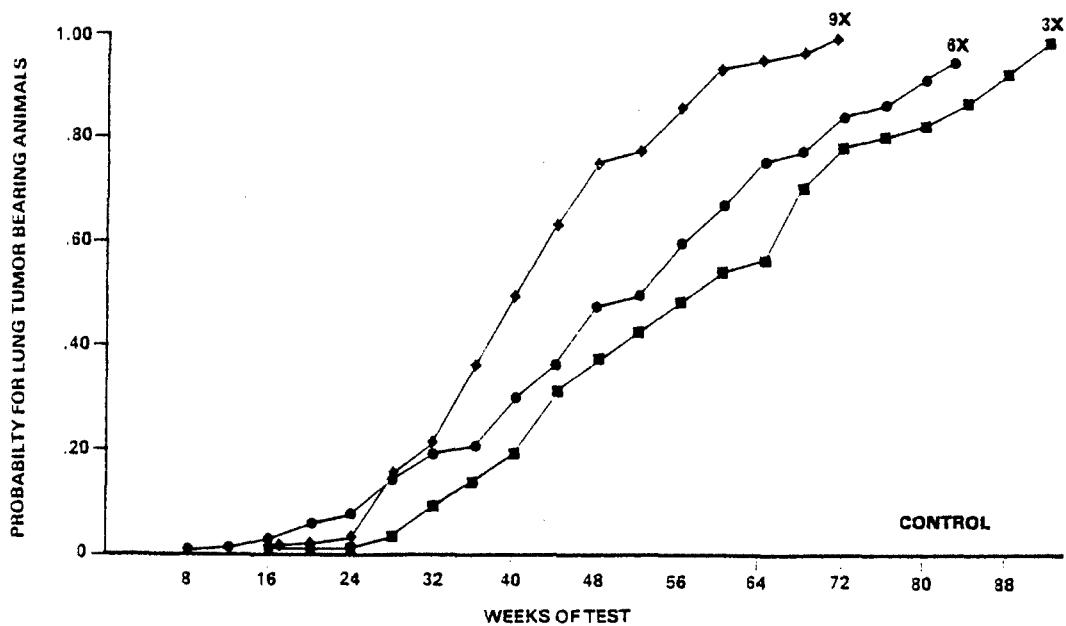


Figure 3. Probabilities for an animal dying of a lung tumor in C3H/Anf Cum mice treated with 3 (■), 6 (●), or 9 (◆) doses of MCA (750, 1500, or 2250 µg MCA total dose, respectively). The number of animals per group are in Figure 1. Over 86%, 72% and 87% of the mice died of lung cancer in the groups treated with 3, 6, or 9 doses of MCA, respectively.

EFFECT OF DIFFERENT CHEMICAL CARCINOGENS

A comparison of the carcinogenic activity of MCA, BP, and BP-7,8-diol is presented in Figure 4. Data are analyzed over a 72 week observation period post chemical treatment. At approximately equal doses, MCA was significantly more carcinogenic than BP and BP-7,8-diol by 24 weeks post treatment. BP-7,8-diol induced a higher probability of death by lung cancer compared to BP by 60 weeks after chemical treatment. During this 72 week observation period, the BP-treated animals expressed no higher probability of lung cancer than the gel-saline treated control animals. By 84 weeks, however, the BP-treated animals were observed to have significantly more lung cancer-related deaths than the control animals (data not shown). Most (~85%) of the lung tumors were AAC rather than SCC in these BP or BP-7,8-diol-treated mice.

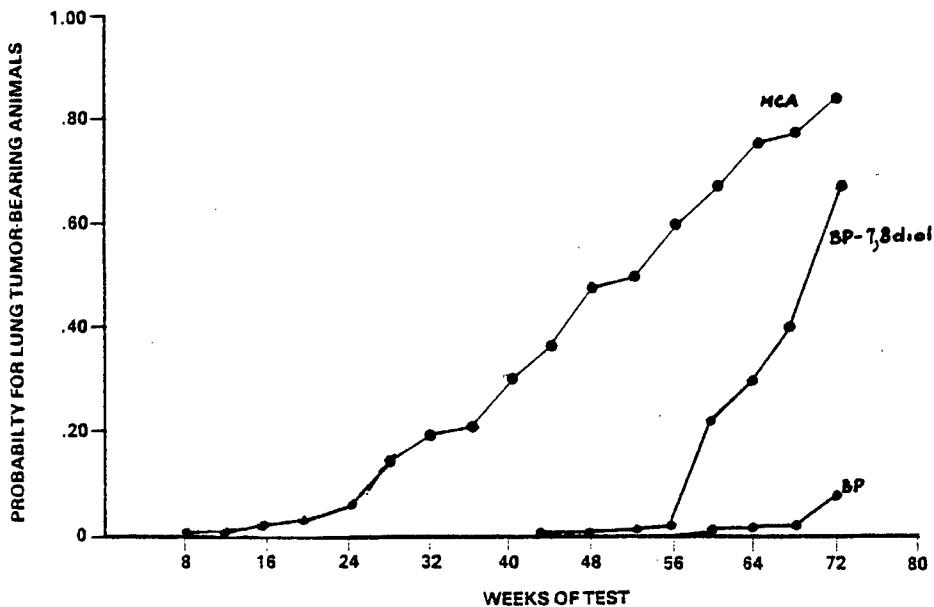


Figure 4. Probabilities for animals dying of a lung tumor in C3H/Anf Cum mice treated with 6 doses of MCA (1500 μ g MCA total), 5 doses of BP-7,8-diol (1250 μ g BP-7,8-diol total), or 5 doses of BP (1250 μ g BP total). The number of animals are 62, 76, and 85 for the MCA, BP-7,8-diol, and BP treated groups, respectively.

DISCUSSION

A lung cancer model system using inbred strains of mice requires knowledge about the: a) natural life expectancy of the mouse strains; b) natural or spontaneous level of expression of lung cancer; c) types of pulmonary tumors which occur; d) sensitivity to chemical carcinogen-induced lung cancer (especially carcinomas); and e) biological behavior of the spontaneous and induced lung tumors. In this study, information concerning spontaneous and chemically-induced lung cancer in C3H/Anf Cum and BC3F1/Cum strains of mice is presented. These studies were performed in mice free throughout their lifetime of infectious disease and of 11 adventitious agents. In particular, these animals were free from two agents which cause lung lesions, Sendai virus and pneumonia virus of mice. The effect of Sendai virus infections on pulmonary carcinogenesis studies has largely been ignored⁽¹³⁾, despite the wide variety of responses altered by infectious Sendai virus⁽⁷⁾. The ability to compare such experimental variables as different chemicals, doses, dose regimens, strains, or species may be completely compromised in animals that had undergone a Sendai virus infection. With the availability of the Sendai vaccine, the problems specific to Sendai virus could be eliminated, but not the problem of adventitious agents in general.

Both of these strains of mice expressed a low spontaneous incidence of lung neoplasia, and these tumors appeared late in the life of the animal. The incidence of lung cancer in control mice is zero during the time at which chemically induced lung cancers appeared. Competing risks were apparent in the old age animals. For example, the higher incidence of mammary cancers in the C3H/Anf Cum strain compared to the BC3F1/Cum strain may have resulted in the C3H/Anf Cum mice dying from mammary cancer before neoplasm of other systems could be expressed.

The lung tumors observed in these studies appeared to be similar in morphology to those reported by Nettesheim and Hammons⁽¹²⁾, Ho et al.⁽⁶⁾, and Yoshimoto et al.⁽²⁰⁾, who have studied MCA and BP induced lung cancers in the inbred strains of mice. The most prevalent tumors observed in either strain were SCC and AAC, with smaller numbers of PDC and ASC (see Table 3 and Figure 1). The types of tumors observed were dependent upon the time at which the animal died after carcinogen treatment. Tumors observed in animals that died less than 40 weeks after carcinogen treatment were almost always SCC (76-93% Table 3 and Figure 1), while tumors in animals that died after 50 weeks were generally AAC (92-100%, Table 3 and Figure 1). These results are very similar to the recent results of Yoshimoto et al.⁽²⁰⁾ who reported that 77-87% of the tumors observed early after intratracheal treatment with BP were SCC, whereas 76-91% of the tumors were adenomas or AAC in the late period of observation (>50 weeks). The results suggest that the different neoplasms may arise from different cell types or that the expression of different neoplasms may be regulated by the carcinogen treatment.

The studies in which different dose levels of MCA were used (see Figure 1) suggested that a larger dose of MCA was required for the induction of SCC rather than AAC. When the dose of MCA was lower, SCC were observed at a lower frequency, while AAC were observed at a higher frequency. SCC seemed to be initiated earlier and caused the death of the animal before AAC could be fully expressed. The mechanism by which higher dose levels of MCA (or longer exposure times) specifically induced the formation of SCC cannot be determined at this time. Analysis of data from a parallel study demonstrates that multiple hyperplasia metaplastic and neoplastic lesions are found in animals sacrificed before 30-40 weeks on test.²

² Lung Tumor Incidence and Progression of Chemically Induced Lung Cancer in BC3F1/Cum Mice. C. J. Henry, L. H. Billups, M. D. Avery, D. R. Dansie, W. C. Hall and R. E. Kouri, 1981, in preparation.

Studies with different chemical carcinogens (Figure 4) show very clearly that this model system and this method of data analysis can effectively discriminate chemicals with different carcinogenic potencies. In terms of carcinogenicity in this model system, MCA > BP-7,8-diol >> BP.

The method of data analysis described here can be used to estimate the discriminating capacity of this lung cancer model system. For example, assuming a population of 50 animals on test, the cumulative probability of an animal dying with a spontaneous lung carcinoma in BC3F1/Cum mice is < 0.02. A total of approximately 5 tumors or greater in the test group of 50 animals would be sufficient to yield a tumor probability which is significantly higher than control values ($p < 0.05$).

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**STRAIN A MOUSE LUNG ADENOMA BIOASSAY FOR
CHEMICAL CARCINOGENS**

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INTRODUCTION

Strain A mice develop a high incidence of primary lung tumors during their lifetime. At 18 to 24 months of age, nearly 100% of strain A mice will have at least one primary tumor per lung and, occasionally, more than one tumor per lung. These tumors are commonly referred to as adenomas. In the gross, or after fixation, the adenomas appear as pearly-white, discrete round nodules, often situated just below the visceral pleura (Figure 1). The tumors are found throughout the lungs; i.e., there is no predilection for side or lobe. With practice, adenomas can be identified and enumerated with the naked eye or under a dissecting microscope when they are a millimeter or less in diameter.

The major determinant for the appearance of lung adenomas in strain A mice is the age of the animal. Older animals have a higher frequency of spontaneous lung tumors than young animals (Table 1). The development of lung adenomas is not related to the sex of the animal although the males of strain A usually have a slightly higher incidence of both "spontaneous" adenomas and chemically-induced adenomas than females. There is no relation between the susceptibility of strain A mice to lung tumors and their susceptibility to "spontaneous" or induced neoplasms in other organs.

The first description of the induction of lung adenomas in mice by carcinogenic agents was that of Murphy and Sturm (1925). They found an increased incidence of lung tumors in mice following the cutaneous painting of coal tar. Shimkin (1940), and Andervont and Shimkin (1941) first applied lung adenomas in strain A mice to quantitative bioassays of chemicals for carcinogenic activity. They reported that the intravenous administration of 3-methylcholanthrene and other polycyclic hydrocarbons to strain A mice led to a significant increase in the frequency of lung adenomas, and the



Figure 1. Induced multiple lung tumors in strain A male mouse 13 weeks after intravenous injection of 1.5 mg of 3-methylcholanthrene. X 3.5

increased tumor response was directly related to the dose of carcinogen. Since these reports, several classes of chemicals including nitrogen mustards (Skimkin et al., 1966), aziridines (Shimkin et al., 1969), carbamates (Shimkin et al., 1969), silylating agents (Stoner et al., 1975), food additives (Stoner et al., 1973), chemotherapeutic drugs (Stoner et al., 1973), organohalides (Poirier et al., 1975; Theiss et al., 1977; Theiss et al., 1979), metals (Stoner et al., 1976; Shimkin et al., 1978), aflatoxin B₁ (Wieder et al., 1968), aminofluorenes (Shimkin and Stoner, 1975), etc., have been examined for carcinogenic activity by the strain A mouse-lung-tumor-induction technique.

TABLE 1. PULMONARY TUMORS IN UNTREATED STRAIN A MICE
AS A FUNCTION OF AGE

Age (months)	Mice (number)		Tumors (% of mice)		Tumors/mouse (Mean No.)	
	Male	Female	Male	Female	Male	Female
2	10	10	0	0	0	0
3	10	10	0	0	0	0
4	20	21	0	4.8	0	0.05
5	20	20	0	0	0	0
6	20	20	0	5	0	0.05
7	20	20	5	5	0.05	0.05
8	34	41	12	7	0.12	0.07
9	44	34	18	21	0.23	0.29
10	39	128	44	23	0.51	0.25
11	55	51	40	37	0.49	0.41
12	70	76	39	36	0.50	0.61
13	51	53	57	40	0.80	0.47
14	76	56	43	45	0.57	0.57
15	49	31	59	53	0.96	0.84
16	43	--	67	--	1.1	----
17	32	18	63	61	1.2	0.89
18	49	37	73	65	1.2	1.6
19	47	18	81	67	1.6	1.1
20	23	20	78	75	1.5	1.8

This discussion describes the procedure used in our laboratory for determining the carcinogenic activity of chemicals by the strain A mouse lung tumor bioassay. In addition, some applications of the bioassay for several classes of compounds are discussed.

MATERIALS AND METHODS

As indicated, the strain A mouse lung tumor bioassay has been applied to several classes of chemical carcinogens. Indeed, the lung tumor reaction was the first evidence of the carcinogenic properties of urethan, isonicotinic hydrazide, and cinnamyl anthranilate (Stoner et al., 1973). Based upon prior experience, a suggested protocol for the bioassay is described.

ANIMALS

Male and female A strain mice, 6 to 8 weeks old, and weighing an average of 18 to 20 grams, are randomly distributed among experimental and control groups. Both A/He and A/Jax mice are suitable substrains for the bioassay since they are equally susceptible to "spontaneous" and chemically-induced lung adenomas.

It is important to use healthy animals previously shown by routine histopathology and serology to be free of pneumonia or other diseases. The animals are housed in groups of five in polycarbonate cages. Commercial grade corn cob bedding is used and food and water are available ad libitum. Hygienic conditions are maintained by twice-weekly changes of the animal cages, and water bottles and cages are sterilized routinely. All chemicals are stored and prepared for injection in a separate room at a distance from the animal quarters.

CHEMICALS

All chemicals are stored at either 4°C or -20°C (depending upon their stability) in the dark. The chemicals are tested for solubility in deionized doubly-distilled water or in tricaprylin (glycerol trioctanoate). Since some commercial lots of tricaprylin contain aldehydes we recommend that the tricaprylin be redistilled before use. Compounds insoluble in water are injected in tricaprylin. The positive carcinogen, urethan, is stored at -20°C in the dark.

All chemicals are weighed out in a chemical fume hood under yellow fluorescent lights. Amber colored bottles with silicon stoppers are used to protect the chemicals from fluorescent light during the injections.

PRELIMINARY TOXICOLOGY

For each chemical under test, a maximum tolerated dose (MTD) is determined. Serial 2-fold dilutions of the chemical are injected intraperitoneally (i.p.) into groups of five mice. The MTD for each chemical is the maximum single dose that all five mice tolerate (survive) after receiving six i.p. injections over a 2-week period. Since the toxic effects of some chemicals are delayed, we routinely hold the animals for 6 to 8 weeks after administering the six doses of the MTD before initiating the chronic bioassay.

BIOASSAYS

Using the preliminary toxicology as a guide, experimental groups are started for each chemical. Three dose levels are used: the MTD; and 1:2 and 1:5 dilutions of the MTD. There are at least 30 mice per dose level, 15 males and 15 females. The animals are weighed every two weeks during the injections and at monthly intervals thereafter.

Each chemical is injected i.p. 3 times weekly for a total of 24 injections. Fewer injections of the more toxic compounds are administered when in the course of the injection period it becomes apparent that the animals will not tolerate the full 24

injection regimen. With known carcinogens such as 3-methylcholanthrene or urethan, a single injection of 1-2 mg is sufficient to elicit a significant increase in lung adenomas.

Two series of baseline controls are maintained during the experimental period. One consists of untreated mice killed along with the treated animals to determine the incidence of spontaneous pulmonary adenomas. The other controls receive injections of either water or tricaprylin (vehicle controls).

Positive control groups consist of animals treated with 2 dose levels of urethan (10 or 20 mg per mouse) to determine whether the tumor response is comparable to that observed in previous studies with strain A mice.

The experiments are terminated 16 weeks after the last injection (24-week bioassay). Treated and control animals are killed by cervical dislocation and their lungs removed and fixed in Tellyesniczky's fluid; the tumors appear as pearly white nodules that can easily be observed on the lung surface. Twenty-four hours after fixation, the tumors on the lung surface are counted and a few taken for histopathologic examination to confirm the histologic appearance of adenoma. The lungs are also examined grossly and microscopically for the presence of other abnormalities such as inflammatory reactions and adenomatosis.

Other organs examined at necropsy for the presence of abnormalities are the kidney, liver, spleen, thymus, salivary and endocrine glands, and the intestine. Grossly abnormal tissues are excised and examined histologically for neoplasms or other reactions.

DATA EVALUATION

The frequency of lung tumors in the chemically-treated groups is compared with that in the vehicle controls by standard Student's t-test. Usually the vehicle control data are not appreciably different for the 2 sexes; therefore, the control data for males and females can be combined and compared to mean tumor values obtained from the treated groups.

The carcinogenic potency of chemicals tested in the lung tumor assay can be compared by determining the dose of each chemical required to produce a minimum carcinogenic response (usually a mean number of 0.8 to 1 adenoma per mouse when compared to controls). This can be done by plotting the mean number of lung tumors versus the log of the molar dose of the chemical. Since a linear dose-response is obtained in the assay, the "carcinogenic index" for each chemical will be the dose at which the response line transects the 0.8 to 1 tumor per mouse level (Zweifel, 1966).

RESULTS

LUNG TUMORS IN CONTROLS

Table 2 gives representative data on the incidence of lung tumors in mice that either received 24 thrice-weekly injections of the 2 vehicles, water or tricaprylin, or a single i.p. injection of urethan (10 or 20 mg/mouse), or they were untreated (Stoner et al., 1973).

TABLE 2. PULMONARY TUMORS IN UNTREATED, VEHICLE-TREATED, AND URETHAN-TREATED STRAIN A MICE^a

<u>Treatment</u>	<u>Duration of experiment (wk)</u>	<u>No. of i.p. injections</u>	<u>Sex</u>	<u>Survivors/initial</u>	<u>Mice with lung tumors (%)</u>	<u>No. of tumors/mouse</u>
Untreated	24	0	M	46/50	22	0.22 ± 0.03 ^b
			F	48/50	17	0.17 ± 0.02
Vehicle Water	24	24	M	30/30	27	0.27 ± 0.05
			F	28/30	37	0.37 ± 0.07
Tricaprylin	24	24	M	77/80	28	0.24 ± 0.03
			F	77/80	20	0.20 ± 0.02
Urethan 10 mg	24	1	M	20/20	100	10.5 ± 2.30
			F	20/20	100	9.1 ± 2.28
Urethan 20 mg	24	1	M	20/20	100	21.8 ± 4.48
			F	19/20	100	19.6 ± 4.20

^a A/Heston mice were either untreated or received 0.1 ml i.p. injections of water, tricaprylin, or the positive carcinogen, urethan. The animals were killed after a total of 24 weeks and their lungs were removed and placed in Tellyesniczky's fluid. After fixation, the surface tumors were counted; the data are expressed as mean number of tumors per mouse. Data from Stoner et al., (1973).

^b Mean ± S.E.

The "spontaneous" incidence of lung tumors in untreated 8 month-old mice (mean of approximately 0.2 tumors per mouse) is relatively constant from experiment-to-experiment. Data from the vehicle controls show that the occurrence of lung tumors is not significantly affected by the injections. Furthermore, the tumor response to the positive carcinogen, urethan, is dose related, with the production of approximately 1 tumor per mg. This response to urethan has been remarkably invariable over 40 years of experience. The lung tumor response to urethan was nearly identical in at least 2 substrains of strain A; i.e., A/He and A/Jax (G. Stoner and M. Shimkin, unpublished data).

LUNG TUMORS IN MICE TREATED WITH CHEMICALS

Table 3 summarizes data on a few representative compounds from different chemical classes that have been tested for carcinogenic activity by the lung tumor response in strain A mice. It is not a complete list of chemicals tested in the bioassay; for a

more complete listing, refer to the review articles of Shimkin and Stoner (1975) and Stoner and Shimkin (1981, in press). As indicated, most chemicals were administered intraperitoneally; however, the polycyclic hydrocarbons were given intravenously. For brevity, only the data from the dose that gave the highest tumor response are given; results from the other doses are omitted.

POLYCYCLIC HYDROCARBONS

Six of the 10 polycyclic hydrocarbons (PAH) tested by Shimkin (1940) and Andervont and Shimkin (1941) produced a significant increase in the lung tumor response relative to controls. On a molar dose basis, 3-methylcholanthrene and dibenz(a,h)anthracene had approximately equal activity for lung tumor production in mice. 7H-dibenzo(cg)carbazole, benzo(a)pyrene, dibenz(a,j)aceanthrylene and dibenz(ah)acridine were less active. A marginal response was produced by 8-methylbenzo(a)phenanthrene and 7-methylbenzo(a)pyrene. 5-Methoxy-7-propylbenz(a)anthracene and benz(a)anthracene were negative for lung tumor production in A strain mice.

CARBAMATES

Twenty-one carbamates were tested for lung tumor production after 12 thrice-weekly i.p. injections (Shimkin et al., 1969). Results from 12 of the carbamates are given in Table 3. Only five compounds other than urethan produced significantly more tumors than the controls and, with the exception of N-hydroxyethylurethan, all were much weaker than the urethan. The methyl ester was entirely negative, indicating that minor changes in chemical structure (such as the removal of a methyl group from ethyl carbamate) can have a marked influence on carcinogenicity. A marginal response was obtained with n-propyl carbamate and N-methylnaphthyl carbamate. On a molar dose basis, some of the PAH are over 1000-fold more potent as inducers of lung tumors than the carbamates.

AZIRIDINES

Eleven aziridine compounds were tested for lung tumor production in strain A mice (Shimkin et al., 1969). Compounds found to be carcinogenic in the assay are given in Table 3. These include two drugs used in chemotherapy: OPSPA and Thio-TEPA. The carbamoyls include chemicals that are more active than the carbamates, but less active than the polycyclic hydrocarbons.

NITROGEN MUSTARDS

The nitrogen mustards include several compounds used as chemotherapeutic agents in disseminated neoplasms in man. Uracil mustard was found to be the most active, with nitrogen mustard and melphalan in the same order of activity as the more active polycyclic hydrocarbons (Shimkin, 1966).

TABLE 3. CHEMICALS TESTED FOR CARCINOGENIC ACTIVITY BY THE STRAIN A MOUSE LUNG ADENOMA BIOASSAY

<u>Compound</u>	<u>Strain</u>	<u>Route of Exposure</u>	<u>Dose (mmole/Kg)</u>	<u>Mean No. Lung Tumors per Mouse</u>	<u>Tumorigenicity</u>
<u>Polyyclic Hydrocarbons</u>					
3-Methylcholanthrene	A	i.v. ^b	0.015	11.0	+
Dibenz(a,h)anthracene	A	i.v.	0.036	31.0	+
7H-Dibenzo(c,g)carbazole	A	i.v.	0.038	5.7	+
Benzo(a)pyrene	A	i.v.	0.400	3.7	+
Dibenz(a,j)aceanthrylene	A	i.v.	0.033	2.7	+
Dibenz(a,h)acridine	A	i.v.	0.036	2.2	+
8-Methylbenzo(c)phenanthrene	A	i.v.	0.042	0.7	-
7-Methylbenzo(a)pyrene	A	i.v.	0.038	0.6	-
5-Methoxy-7-propylbenz(a)-anthracene	A	i.v.	0.033	0.1	-
Benz(a)anthracene	A	i.v.	0.044	0.2	-
<u>Carbamates</u>					
Ethyl(urethane)	A	i.p.	26.93	24.5	+
N-Hydroxyethyl	A	i.p.	22.83	18.6	+
Allyl	A	i.p.	2.75	1.3	+
N-Cyanoacetylethyl	A	i.p.	15.37	4.0	+
N-Acetylethyl	A	i.p.	18.30	3.2	+
Isopropyl	A	i.p.	23.27	1.5	+
n-Propyl	A	i.p.	24.21	0.8	-
Benzyl	A	i.p.	15.87	0.2	-
Phenyl	A	i.p.	17.50	0.5	-
g-Chloroethyl	A	i.p.	19.42	0.4	-
n-Butyl	A	i.p.	20.48	0.3	-
Methyl	A	i.p.	31.95	0.1	-
<u>Aziridines</u>					
3,4-Dichlorophenyl-N-carbamoyl	A	i.p.	0.10	1.3	+
m-Chlorophenyl-N-carbamoyl	A	i.p.	1.22	5.0	+
Phenyl-N-carbamoyl	A	i.p.	1.48	2.0	+
Cyclohexyl-N-carbamoyl	A	i.p.	1.43	1.0	+
Bis(1-aziridinyl)morpholino-phosphine sulfide (OPSPA)	A	i.p.	0.52	2.0	+
Tris(1-aziridinyl)phosphine sulfide ("Phio-TEPA")	A	i.p.	0.11	1.7	+
2,5-Bis(1-aziridinyl)-3,6-bis(2-methoxyethoxy)-p-benzoquinone	A	i.p.	0.09	2.9	+
<u>Nitrogen Mustards</u>					
Uracil mustard	A	i.p.	0.04	20.3	+
Nitrogen mustard	A	i.p.	0.02	2.8	+
Melphalan	A	i.p.	0.06	4.0	+
Chloroquine mustard	A	i.p.	0.04	1.4	+
Quinacrine ethyl mustard	A	i.p.	0.06	1.3	+
Benzimidazole mustard	A	i.p.	0.36	3.6	+
Mannitol mustard	A	i.p.	0.38	2.4	+
Chlorambucil mustard	A	i.p.	0.49	5.1	+
Aniline mustard	A	i.p.	0.86	5.5	+
Cyclophosphamide	A	i.p.	0.52	1.3	+
Naphthylamine mustard	A	i.p.	4.48	2.0	+
Mannitol myleran	A	i.p.	14.20	3.2	+
Quinacrine propyl mustard	A	i.p.	0.003	0.4	-
Chloroquine mustard pamoxate	A	i.p.	0.09	0.7	-
<u>Organohalides</u>					
1. <u>Iodides</u>					
n-Propyl iodide	A	i.p.	17.6	0.70	±
n-Butyl iodide	A	i.p.	13.1	0.63	±
sec-Butyl iodide	A	i.p.	32.6	0.63	±
i-Propyl iodide	A	i.p.	35.2	0.58	±
Methyl iodide	A	i.p.	0.3	0.55	±
Ethyl iodide	A	i.p.	38.4	0.15	-
tert-Butyl iodide	A	i.p.	2.7	0.42	-
2. <u>Bromides</u>					
Bromoform (tribromomethane)	A	i.p.	4.3	1.13	+
sec-Butyl bromide	A	i.p.	43.7	1.15	+
2-Bromoethanol	A	i.p.	1.2	0.79	+
tert-Butyl bromide	A	i.p.	43.7	0.78	+
i-Butyl bromide	A	i.p.	43.7	0.75	+
Bromodichloromethane	A	i.p.	14.6	0.85	-
3-Bromopropylamine-HBr	A	i.p.	5.3	0.56	±
2-Bromobutyric acid	A	i.p.	1.4	0.53	-
Butyryl bromide	A	i.p.	1.6	0.45	-
3. <u>Chlorides</u>					
2-Chloro-N,N-dimethyl-amine-HCl	A	i.p.	12.5	1.30	+
sec-Butyl chloride	A	i.p.	35.0	1.20	+
tert-Butyl chloride	A	i.p.	65.0	1.00	+
3-Chloropropionic acid	A	i.p.	6.7	0.90	+
3-Chlorobutyric acid	A	i.p.	9.6	0.89	+
1,2-Dichloroethane	A	i.p.	24.2	0.75	-

TABLE 3. CHEMICALS TESTED FOR CARCINOGENIC ACTIVITY BY THE STRAIN A MOUSE LUNG ADENOMA BIOASSAY (CONTINUED)

Compound	Strain	Route of Exposure	Dose (mmole/Kg)	Mean No. Lung Tumors per Mouse	Tumorigenicity
Hexachlorobenzene	A	i.p.	3.4	0.75	-
4-Chloro-1-butanol	A	i.p.	33.6	0.70	+
Ethyl chloroacetate	A	i.p.	24.0	0.61	±
Benzyl chloride	A	i.p.	11.8	0.50	-
1,1,2,2-Tetrachloro-ethylene	A	i.p.	57.8	0.50	-
1,1,2,2-Tetrachloroethane	A	i.p.	21.4	0.50	-
Chloroform	A	i.p.	16.2	0.36	-
Hexachlorocyclohexane	A	i.p.	6.1	0.20	-
Metal Salts					
Lead (II) succinate	A	i.p.	0.19	1.47	+
Nickelous (II) acetate	A	i.p.	1.45	1.26	+
Manganous sulfate	A	i.p.	3.91	1.20	+
Molybdenum (III) trioxide	A	i.p.	32.99	1.13	+
Iron (II) 2,4-pentanedione	A	i.p.	2.95	0.60	-
Vanadium (III) 2,4-pentanedione	A	i.p.	0.34	0.79	-
Zinc (II) acetate	A	i.p.	1.64	0.78	-
Cupric (III) acetate	A	i.p.	0.99	0.56	-
Cobalt (III) acetate	A	i.p.	2.01	0.79	-
Chromium (III) sulfate	A	i.p.	6.12	0.63	-
Calcium (II) acetate	A	i.p.	7.59	0.58	-
Stannous chloride	A	i.p.	6.33	0.50	-
Cadmium (II) acetate	A	i.p.	0.05	0.40	-
Chemotherapeutic Drugs					
Triethylene melamine	A	i.p.	0.02	26.0	+
Estradiol mustard	A	i.p.	2.01	4.95	+
Phenesterin	A	i.p.	18.61	3.90	+
Mannitol myleran	A	i.p.	0.38	2.40	+
Isophosphamide	A	i.p.	5.00	1.83	+
β-Deoxythioguanosine	A	i.p.	0.58	1.00	+
Imidazole mustard	A	i.p.	5.34	1.00	+
1-Propanol-3,3'-imino-dimethanesulfonate	A	i.p.	3.37	1.00	+
Dapsone	A	i.p.	5.29	0.87	+
Dibenzyline	A	i.p.	0.29	0.79	±
Pyrimethamine	A	i.p.	0.50	0.78	±
5-Azacytidine	A	i.p.	0.37	0.73	±
Phenformin	A	i.p.	7.32	0.53	-
Phenazopyridine	A	i.p.	6.20	0.50	-
ICRF-159	A	i.p.	9.33	0.53	-
Emetine	A	i.p.	0.38	0.50	-
Tolbutamide	A	i.p.	177.58	0.45	-
Adriamycin	A	i.p.	0.07	0.40	-
Potassium arsenite	A	i.p.	0.30	0.400	-
Aminopterin	A	i.p.	0.004	0.300	-
Acronycine	A	i.p.	8.10	0.300	-
Myleran	A	i.p.	24.39	0.300	-
Phenformin	A	i.p.	7.32	0.300	-
Cortisone	A	i.p.	3.45	0.300	-
Stilbamidine	A	i.p.	2.20	0.200	-
Silyating Agents					
Trimethylchlorosilane	A	i.p.	9.17	1.79	+
N-acetylimidazole	A	i.p.	0.91	1.75	+
N-(Trimethylsilyl)-imidazole	A	i.p.	7.14	1.50	+
1-Ethyl-3-p-tolyltriazene	A	i.p.	0.61	1.50	+
N,O-Bis(trimethylsilyl)-acetamide	A	i.p.	4.93	1.15	+
1,3-Bis(chloromethyl)-1,1,3,3-tetramethyldisilazane	A	i.p.	0.87	1.00	+
Hexamethyldisilazane	A	i.p.	6.25	0.60	-
tert-Butyldimethyl-chlorosilane	A	i.p.	6.62	0.59	-
N-Trimethylsilyl-acetamide	A	i.p.	0.76	0.53	-
N-Methyl-N-trimethylsilyl-trifluoroacetamide	A	i.p.	1.00	0.45	-
Miscellaneous Chemicals					
2-Amino-5-azotoluene	A	i.p.	18.70	4.1	+
2-Acetylaminofluorene	A	i.p.	27.00	2.3	+
N-Hydroxy-2-acetylaminofluorene	A	i.p.	25.00	1.9	+
Aflatoxin B ₁	A	i.p.	0.70	5.6	+
Fluorodeoxyuridine	A	i.p.	0.20	0.3	-
Bromodeoxyuridine	A	i.p.	0.16	0.4	-
Iododeoxyuridine	A	i.p.	0.14	0.4	-
Bis(chloromethyl) ether	A	inhal.	25.87	2.9	+

a For brevity, only data from the dose that gave the highest tumor response are given; results from the other doses are omitted.

b i.v. = intravenously; i.p. = intraperitoneally; inhal. = inhalation

ORGANOHALIDE COMPOUNDS

Data from some of the organohalide compounds tested for carcinogenicity in strain A mice were taken from three studies by Poirier et al. (1975), Theiss et al. (1977), and Theiss et al. (1979). Some of these chemicals are contaminants in United States drinking waters (Theiss et al., 1977). In the three studies, different mean tumor values were obtained for the vehicle controls. Therefore, some compounds such as methyl iodide and *n*-propyl iodide were marginally active when eliciting responses as low as 0.55 to 0.70 tumors per mouse, and other compounds such as hexachlorobenzene, dichloromethane and bromodichloromethane were negative when producing responses as high as 0.75 to 0.94 tumors per mouse. As discussed by Poirier et al. (1975), the results with the organohalides indicated that carcinogenicity was related to the structure of the compound. Usually, chemicals with primary structures did not produce a significant increase in the lung tumor response when compared to controls, whereas those with secondary or tertiary structures were positive. However, there were exceptions. For example, the primary halide, methyl iodide, was positive, whereas tert-butyl iodide was negative. On a molar dose basis, the most active alkyl halide was methyl iodide.

Six of the organohalides were carcinogenic in other experimental systems yet were negative for lung tumor production in strain A mice. Chloroform (U.S. National Cancer Institute Report, 1976), tetrachloroethylene (Kylin et al., 1965), 2-chloroethyl ether (Innes et al., 1969), and hexachlorocyclohexane (Nagaski et al., 1971) are all hepatotoxins that elicit a carcinogenic effect primarily on the liver. Benzyl chloride (Druckrey et al., 1970) and ethyl bromoacetate (VanDuuren et al., 1974) produced sarcomas on subcutaneous injection into animals.

METALS

The intraperitoneal administration of lead (II) subacetate, nickelous (II) acetate, manganous sulfate and molybdenum (III) trioxide led to a significant increase in the lung tumor response in A mice (Stoner and Shimkin, 1976). The activity of nickelous acetate and lead subacetate might have been expected since these compounds have been shown to produce tumors in other systems. Moreover, nickel is thought to be carcinogenic for the nasal cavity, paranasal sinuses and lung of man (Hueper, 1971). The carcinogenic activities of manganese and molybdenum are not well documented in the literature. However, manganous chloride has been shown to hasten the appearance of lymphosarcoma in mice (DiPaolo, 1964). Metals that were negative for lung adenoma production in strain A mice but positive in other experimental systems are cadmium, chromium, cobalt, iron and zinc (Furst and Haro, 1969; Sunderman, 1971). If administered at higher doses, these metals may have induced lung tumors in strain A mice.

FOOD ADDITIVES

Forty-one food additives were tested for lung tumor induction in strain A mice (Stoner and Shimkin, 1973). Forty of the 41 food additives were found to be negative; therefore, these compounds are not listed in Table 3. The food additives were selected from the GRAS (generally recognized as safe for human consumption) list either randomly or because they contained double bonds, keto groups, several methoxy groups on a benzene ring, or other structures similar to those of known carcinogens. Cinnamyl anthranilate was the only food additive that produced a significant increase in the lung tumor response relative to tri-caprylin controls. The carcinogenicity of cinnamyl anthranilate may have been due to the interaction of both the cinnamyl and anthranilic acid moieties since cinnamyl alcohol, cinnamyl aldehyde and methyl anthranilate were negative. In a recent publication by Ward et al. (1980), cinnamyl anthranilate was reported to induce liver tumors in mice.

CHEMOTHERAPEUTIC DRUGS

The chemotherapeutic agents listed in Table 3 were tested for lung tumor production by Shimkin et al. (1966) and Stoner et al. (1973). The carcinogenic responses obtained with several of these agents was not unexpected since they are capable of alkylating DNA. On a molar dose basis, uracil mustard (listed under the nitrogen mustards) was considerably more active than either imidazole or estradiol mustards. The enhanced activity of uracil mustard can most probably be attributed to its role as an analog of uracil and its consequent incorporation into RNA as suggested by Abell et al. (1965).

Shimkin et al. (1966) reported that mannitol Myleran was carcinogenic for lung tumor induction in strain A mice. However, Myleran was negative at a 10-fold higher dose than used for mannitol Myleran (Stoner et al., 1973). Since the activity of mannitol Myleran is probably due to the Myleran moiety, the negative result for Myleran was unexpected, and the compound should be re-tested.

SILYLATING AGENTS

The silylating agents are used extensively in many laboratories for the conversion of nonvolatile compounds to volatile materials for gas chromatographic determinations. These reagents are highly reactive and introduce a trimethylsilyl group into otherwise nonvolatile molecules, thus rendering them volatile. Stoner et al. (1975) found that 6 of 10 silylating compounds were positive for lung tumor production in strain A mice. Because of their alkylating ability, it was not surprising that these compounds were active.

It is difficult to assign definitive structure-activity correlations with the silylating agents. However, compounds in which the trimethylsilyl group was attached only to an amide or amino nitrogen were generally inactive (hexamethyldisilazane, N-methyl-N-trimethylsilyl trifluoroacetamide, N-trimethylsilylacetamide). If a chlorine was attached to the trimethylsilyl moiety (trimethylchlorosilane), the compound was active. Replacing the methyl group in the trimethylsilyl moiety by the hindered tert-butyl group led to loss of activity (tert-butyldimethylchlorosilane). The activity of 1-ethyl-3-p-tolyltriazene was expected since it is an analog of some triazenes which Druckrey et al. (1967) found to be potent and selective carcinogens.

MISCELLANEOUS CHEMICALS

Four carcinogenic chemicals; i.e., 2-amino-5-azotoluene, (Andervont, 1941), 2-acetylaminofluorene, N-hydroxy-2-acetylaminofluorene (Shimkin and Stoner, 1975), and aflatoxin B₁ (Weider et al., 1968), whose predominant biological effect is hepatotoxicity and hepatocarcinogenicity in the rat were positive for lung tumor induction in strain A mice. Aflatoxin B₁ was the most active of the four in producing lung tumors, and on a molar dose basis was about 10 times as active as urethan. Aminoazotoluene was intermediate in activity, and the aminofluorene compounds were the least active.

The three nucleotide base analogs (fluorodeoxyuridine, bromodeoxyuridine and iododeoxyuridine) were found to be without significant carcinogenic activity for mouse lung (Poirier et al., 1975). Previous studies have reported that neither bromodeoxyuridine nor iododeoxyuridine possesses significant carcinogenic activity (Hadidian et al., 1968). Leong et al. (1971) reported the production of lung tumors in strain A mice by the chronic inhalation of bis(chloromethyl) ether.

RELATIVE CARCINOGENICITY OF SELECTED COMPOUNDS

Table 4 illustrates the use of the method of Zweifel (1966) (Materials and Methods) to estimate the relative potency of some of the positive compounds selected from Table 3 to produce lung adenomas in strain A mice. 3-Methylcholanthrene, uracil mustard and dibenz(ah)anthracene are of approximately equal activity and of the highest potency. Benzo(a)pyrene is approximately one-tenth as active as 3-methylcholanthrene, uracil mustard and dibenz(ah)anthracene. Aflatoxin B₁, a very potent inducer of liver tumors in rats, is much less active than benzo(a)pyrene. Likewise, the two hepatocarcinogens, 2-acetylaminofluorene and N-hydroxy-2-acetylaminofluorene, are weakly active for lung tumor production in mice. The food additive, cinnamyl anthranilate, was one of the least active compounds tested.

TABLE 4. RELATIVE CARCINOGENIC ACTIVITY OF SELECTED CHEMICALS FOR LUNG TUMOR PRODUCTION IN STRAIN A MICE^a

<u>Compound</u>	<u>Total Dose (moles/Kg)</u>	<u>Mice with LT/ No. of Mice</u>	<u>Mean No. of LT/Mouse</u>	<u>Dose for 1 LT^b Response (μmoles/Kg)</u>
3-Methylcholanthrene	15	15/15	11.0	0.9
Uracil mustard	38	30/30	20.3	1
Dibenz(a,h)anthracene	36	10/10	31.0	1
Triethylene melamine	25	24/28	26.0	8
Benzo(a)pyrene	40	10/10	3.7	9.5
Dibenz(ah)acridine	36	11/12	2.0	18
3,4-Dichlorophenyl-N- carbamoyl	101	12/15	1.3	85
Aflatoxin B ₁	700	14/14	5.6	120
Thio-TEPA	499	16/20	1.5	143
Estradiol mustard	2,028	19/19	4.9	179
Ethyl carbamate (urethan)	26,933	12/12	24.5	1,963
N-Acetylethyl carbamate	18,203	15/16	3.2	2,371
2-Amino-5-azotoluene	18,700	15/15	4.1	4,560
Trimethylchlorosilane	9,200	11/14	1.8	5,350
2-Acetylaminofluorene	27,000	20/21	2.3	11,739
N-Hydroxy-2-acetylaminofluorene	25,000	16/19	1.9	13,158
Cinnamyl anthranilate	47,000	11/15	2.4	19,800
Isopropyl carbamate	23,275	8/10	1.5	26,300

^a Data taken from Shimkin and Stoner (1975)

^b LT = Lung Tumor

DISCUSSION

This report describes the basic protocol of the strain A mouse lung adenoma bioassay and provides summary data on its application for the testing of selected compounds from different chemical classes for carcinogenic activity. Omitted from the table were studies with "crudes" such as tobacco tar and coal tar, and of compounds for which insufficient quantitative data were available. Also omitted were data from investigations conducted in other strains of mice. For example, Mirvish and Kaufman (1970) reported the induction of lung adenomas in SWR mice given 2 i.p. injections of several nitrosamines, and Roe et al. (1967) reported the induction of lung adenomas in SWR mice with hydrazine compounds. We refer the reader to the review articles of Shimkin and Stoner (1975) and Stoner and Shimkin (1981, in press) for complete discussions of the use of the lung tumor response in mice for carcinogenesis testing, and of the effects of both inhibitors (e.g. butylated hydroxyanisole) and promoters (e.g.; phorbol oil) of carcinogenesis on the tumor response. In addition, the review of Shimkin and Stoner (1975) describes the effects of various immunologic factors, viruses, germ-free state, atmospheric oxygen, etc., on the lung tumor response to chemicals.

The major criterion for a positive carcinogenic response in the lung adenoma assay is a statistically significant increase in the average number of lung tumors in chemically-treated mice versus the vehicle-injected controls. Generally, when 30 mice are used per concentration of test compound, levels of statistical significance ($p = <0.05$) may be reached when a mean of 0.7 to 0.8 tumor per mouse is observed in chemically-treated mice and compared to a mean of 0.2 to 0.3 lung tumors among the controls at the age of 8 to 9 months. However, as indicated in Table 3, some compounds were positive when inducing a mean of 0.5 to 0.6 tumors per mouse because the vehicle-control data were lower than a mean of 0.2 to 0.3 tumor per mouse. These should be considered very marginal responses. In fact, in our experience with the assay, compounds should be considered positive if the following conditions are met: (a) the mean number of lung tumors in the test animals is significantly increased, preferably to 1 or more per mouse, (b) there is a dose-response relationship, and (c) the mean number of lung tumors in the vehicle-injected and untreated controls is approximately the anticipated number for untreated mice of the same age.

To our knowledge, there have been few "false positives" in the lung adenoma bioassay. Molybdenum trioxide and manganous sulfate may be examples of false positives. Nearly all other compounds that induced lung tumors in A mice have also evoked a neoplastic response in other experimental animal systems and/or demonstrated mutagenic activity in various short-term assays. In fact, even the weakly-active inducer of lung tumors, cinnamyl anthranilate (Table 4; Stoner et al., 1973) was recently shown in a long-term study (Ward et al., 1980) to induce liver tumors in mice and is now considered a bona fide carcinogen.

A few compounds that produced tumors in other experimental animal test systems were negative for lung tumor production in strain A mice. However, in some instances, these compounds have been only marginally active in the other systems; e.g., saccharin. Several potent hepatocarcinogens in rats; e.g., aflatoxin B₁, acetylaminofluorene and N-hydroxyacetylaminofluorene, were more weakly active for lung tumor production in strain A mice. Studies of the pharmacokinetics of distribution and metabolism of these compounds in the liver and lung might explain the differences in their carcinogenic potency.

Pulmonary tumors in mice have attracted cancer investigators in many laboratories throughout the world. The criticisms of the bioassay are that the adenoma is a "benign" tumor and has no counterpart in human neoplastic pathology, and that the numerical increase and earlier appearance of the tumor merely accelerates a process already present in the animal rather than being a truly inductive process. In answer to these criticisms, some adenomas progress to adenocarcinomas with invasion of the surrounding lung if the mice are maintained for sufficient periods (18-24 months).

after administration of the carcinogen, and a small percentage of the adenocarcinomas metastasize to other organ sites (Kimura, 1971). Therefore, not all primary lung tumors can be classified as benign tumors. During the six-month bioassay, most of the tumors have simply not progressed beyond the adenoma stage. As to the second criticism, i.e., that the adenoma has no close counterpart in human pathology, it is known that some alveolargenic carcinomas in man are derived from the type 2 alveolar epithelial cells. The predominance of human lung tumors in the bronchus is probably due to its higher exposure to carcinogens and, in particular, those carcinogens in tobacco smoke. Regarding the question of induction versus acceleration of lung tumors, the administration of a carcinogen such as methylcholanthrene at a dose of 1 mg per mouse can lead to the production of a mean of 40 lung adenomas per mouse within 6 months or less. We have never observed more than an average of 0.2 to 0.5 lung tumor per mouse in controls at 6 months, or 1 to 2 tumors per mouse in controls at 18 to 20 months. Moreover, histologic examination of the lungs of control mice at 6 to 18 months does not reveal evidence of numerous foci of small, developing tumors. Therefore, it is likely that the production of lung adenomas in strain A mice by carcinogenic agents is an inductive rather than an accelerative process.

In our opinion, the strain A mouse lung tumor bioassay deserves further investigation in the following areas: (1) The distribution and metabolism of carcinogens in the whole lung and in specific lung cell types such as the type 2 alveolar epithelial cells; (2) Examination of the lung adenoma bioassay with respect to its ability to detect promoting agents, and; (3) Quantitative studies of the potential inhibitory, additive or synergistic effects of administering more than one chemical to the animals (this seems particularly relevant in view of the fact that humans are constantly exposed to a large number of environmental chemicals).

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METABOLISM OF BENZO(A)PYRENE IN CULTURED HUMAN BRONCHUS*

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INTRODUCTION

Lung cancer is a major cause of cancer deaths in many countries. Based on clinical, experimental, epidemiologic and autopsy data, cigarette smoking has been identified as the major risk factor in the development of lung cancer (Royal College of Physicians, 1971). The majority of lung cancers in man are bronchiogenic carcinomas which are thought to arise from a metaplastic squamous differentiation of the large bronchi. Polycyclic aromatic hydrocarbons, such as benzo(a)pyrene (PB), found in cigarette smoke and environmental pollutants, have been associated with lung cancer in man (National Academy of Science, 1972). Since most chemical carcinogens have to be metabolically activated in the body before exerting their carcinogenicity (Miller and Miller, 1966), it is important to study the metabolic fate of benzo(a)pyrene in the human bronchus.

Recent developments in the maintenance and growth of viable human epithelial tissues and cells *in vitro* have made possible the study of chemical carcinogenesis directly in human tissue. Initial studies have shown that cultured tissues and whole cells from several human organs including lung (Harris et al., 1976; Cohen et al., 1976; Harris et al., 1977; Autrup et al., 1978; Stoner et al., 1978), colon (Autrup et al., 1980), esophagus

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(Harris et al., 1979), pancreatic duct (Harris et al., 1977), mammary gland (Grover et al., 1980), endometrium (Mass et al., 1981), skin (Kuroki et al., 1980) and blood monocytes (Lake et al., 1977) are capable of metabolizing chemical carcinogens. In the present report, we summarize data obtained from studies of the metabolism of BP in cultured explants of human bronchus. The data indicate that: a) the pathways for the metabolic activation of BP in cultured human bronchus are similar to those in other human and animal tissues; b) there are significant variations among individuals in the extent of binding of BP metabolites to bronchial cell DNA; and c) the adducts formed between BP and DNA in cultured human bronchus are similar to those identified by previous workers in explants of human bronchus and in other human and animal tissues.

MATERIALS AND METHODS

CHEMICALS

BP (generally tritium labeled, 30-40 Ci/mmol) was purchased from Amersham Corporation, Arlington Heights, Illinois, and diluted to a specific activity (SA) of 3-5 Ci/mmol with unlabeled BP (Eastman Organic Chemicals, Rochester, New York). The compounds were purified and judged to be chemically and radiochemically pure by high-pressure liquid chromatography (HPLC). The SA was calculated from the molar absorptivity of BP ($\epsilon_{\text{MeOH}} = 60,000$) and determination of radioactivity by liquid scintillation counting. Purified [^3H]BP was dissolved in a mixture of dimethylsulfoxide (DMSO):methanol (4:1) and stored at 4°C at a concentration of 0.1 mM before addition to the explant cultures.

Authentic metabolites of BP were obtained from the Chemical Repository of the National Cancer Institute, Bethesda, Maryland. All compounds were stored at -20°C in the dark. Hydroxylapatite (HTP-DNA grade) was purchased from Bio-Rad, Richmond, California and stored at 4°C until used. All solvents for chromatography were HPLC grade and obtained from Fisher Scientific, Pittsburgh, Pennsylvania. DMSO was purchased from Burdick-Jackson, Muskegon, Michigan.

TISSUE SPECIMENS

Grossly normal bronchial tissues were taken from 16 patients at autopsy within 2-6 hours of the time of death. The age, sex, and time after the patient's death before tissues were placed in culture are given in Table 1. Tissues from patients with sepsis, metastatic cancer, serum hepatitis or a history of tuberculosis were not used in these studies. The tissues were immersed in ice-cold L-15 medium immediately after removal from the patient and transported to the laboratory. Using aseptic conditions, the bronchial specimens were trimmed of adherent lung tissue. Pieces

of tissue from each specimen were fixed in a phosphate buffered solution containing 4% formaldehyde and 1% glutaraldehyde (4F1G) (McDowell and Trump, 1976), embedded in Epon and assessed by light microscopy after staining with toluidine blue.

TABLE 1. SOURCE OF HUMAN BRONCHI

Case Identification Number	Age (Yr.)	Sex	Hours After Death Until Specimen Culture
5	15	M	6
9	22	F	2.5
14	55	F	2.5
15	10	M	3
19	50	F	2.5
22	33	M	4
23	29	M	6
24	55	M	3
31	55	M	5
33	75	M	2.5
34	42	F	3.5
*35	--	-	5
73	76	M	5
77	63	M	4.5
98	56	M	2
100	25	M	3
103	67	F	3

*The age and sex of case number 35 was not recorded.

EXPLANT CULTURE CONDITIONS

Bronchial samples were cut into approximately 0.5 x 0.5-cm pieces and placed on the etched surface of 60-mm plastic tissue culture dishes (one piece/dish) with the epithelium oriented toward the gas-liquid interface (Trump et al., 1974). The number of bronchial pieces obtained from each case ranged from 4 to 78. To each culture dish was added two and one-half ml of PFMR-4 medium (Lechner et al., 1980) supplemented with crystalline bovine insulin (1 μ g/ml, Eli Lilly, Indianapolis, Indiana), hydrocortisone hemisuccinate (35 ng/ml, Upjohn Company, Kalamazoo, Michigan), β -retinyl acetate (0.1 μ g/ml, Hoffmann LaRoche, Nutley, New Jersey), gentamicin (50 μ g/ml), penicillin G (100 U/ml), kanamycin (100 μ g/ml), and amphotericin B (2.5 μ g/ml). The cultures were maintained in an air-tight chamber at 36.5°C in an atmosphere of 50% O₂-45% N₂-5% CO₂ at a pressure of 3 psi. The chamber was placed on a rocker platform (Bellco Glass Company, Vineland, New Jersey) and rocked in the dark at approximately 5 cycles/min so that the tissues were alternately exposed to the

medium and to the atmosphere. Every 2 days the medium was replaced and the atmosphere of the cultures was restored by flushing the chamber with the gas mixture at 5 liters/min for 5 minutes.

INCUBATION WITH [³H]BP AND SAMPLE PREPARATION

On the third day of explant culture [³H]BP (SA 3-5 Ci/mmol) was added to the culture medium at a concentration of 1 μ M. After incubation with carcinogen for 24 hours, the medium and tissues were separated and the media were stored immediately at -20°C. The explants were rinsed twice with cold phosphate buffered saline (PBS) containing 0.1% glucose. The bronchial mucosa containing epithelial and mesenchymal cells was stripped from the supporting structures with forceps and stored at -20°C. Cells from 4 to 8 cultures were pooled for each experimental variable. Incubations of medium with [³H]BP but without the explants served as controls.

PREPARATION OF BP-DNA ADDUCT STANDARDS

Synthetic BP-DNA adducts were prepared by a method similar to that of Remsen et al. (1977). Briefly, 0.95 mg of calf thymus DNA (P & L Biochemicals, Milwaukee, Wisconsin) was dissolved in 1 ml of water and treated with 0.4 ml of methanol followed by addition of 93 μ l of a solution of anti-7 β ,8- α dihydroxy-9 α ,10 α -epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (anti-BPDE, or BPDE-I) in DMSO (7 mg/ml). The reaction mixture was incubated at 37°C for 3.5 hours, followed by addition of 150 μ l of saturated NaCl and precipitation of the DNA by addition of one volume of 2-ethoxyethanol. The BP-modified DNA was washed twice with absolute ethanol, and twice with anhydrous ether and stored at -20°C until used. A portion of this material was hydrolyzed by enzymatic procedures (see below) and extracted with 1 volume of water-saturated n-butanol. Following removal of the n-butanol under N₂ the residue was dissolved in 50% methanol in H₂O and chromatographed as described below. The major UV peaks eluting at 63% (peak fraction 27) and 65% (peak fraction 31) methanol were collected and their UV-spectra were identical to those reported by Jeffrey et al. (1977) for BP-DNA adducts; i.e. 7S-BPDE-I-dG (peak fraction 27) and 7R-BPDE-I-dG (peak fraction 65). These adducts were dissolved in methanol, stored at -20°C, and used as chromatographic standards.

ISOLATION OF [³H]BP BOUND TO DNA

Bronchial explants were homogenized in 2 ml 0.05 M sodium phosphate buffer, pH 6.5, containing 0.01 M EDTA and 0.01 M EGTA, using a Tissumizer (Tekmar, Cincinnati, Ohio) at maximum speed for 30 seconds. The homogenates were made 1.5% (v/v) in sodium dodecyl sulfate, treated with 0.2 mg autodigested pronase B (Calbiochem, LaJolla, California) and incubated for 2.5 hours at 37°C with mixing. The samples were made 7% (w/v) in 4-aminosalicylate-NaCl (6:1) and extracted for 1 hour with 1 volume of phenol reagent

(Kirby, 1965), after which the phases were separated by centrifugation (40 min at 12,000 X g) and the aqueous phase removed. The phenol phase was back-extracted with 1 ml buffer and the resulting aqueous phases pooled. Hydroxylapatite (0.5 g), suspended in 2 ml 0.05 M sodium phosphate buffer, pH 6.5, was added to the pooled aqueous layers in a 15 ml polystyrene centrifuge tube. The gel was washed four times with 10 ml volumes of a solution of 8 M urea in 0.12 M sodium phosphate buffer, pH 6.5, in a batch procedure, followed by two 5 ml washes of 0.05 M sodium phosphate buffer. The DNA was then eluted from the hydroxylapatite with two 1.5 ml washes of 0.55 M sodium phosphate buffer, pH 6.5. The amount of DNA present in the aqueous samples was determined fluorimetrically after reaction of an aliquot (150 μ l) with 3,5-diaminobenzoic acid (Kissane and Robbins, 1958). The radioactivity associated with the DNA was determined by liquid scintillation counting in PCS (Amersham, Arlington Heights, Illinois) after digestion with perchloric acid. The binding levels were then calculated and expressed as μ moles BP/mole deoxynucleotide.

BP-DNA ADDUCT CHROMATOGRAPHY

The purified BP-modified DNA solutions were pooled, dialyzed, dried and then redissolved in 2 ml 0.1 M Tris buffer, pH 7, containing 10 mM MgCl₂, and 1 mM ZnCl₂. The DNA was hydrolyzed enzymatically to the nucleoside level with DNase I, snake venom phosphodiesterase, and alkaline phosphatase (Worthington, Bedford, Maryland) at 37°C for 16 hours. The BP-DNA adducts were extracted from the hydrolysate into an equal volume of n-butanol (water saturated), the butanol layer removed, and dried under N₂ at 37°C. The resulting residue was redissolved in 1 ml 50% MeOH in H₂O for HPLC.

Samples were checked by thin-layer chromatography on cellulose (Eastman No. 13254) with 1 M ammonium acetate, pH 7.5:ethanol (33:67) against deoxynucleoside and deoxynucleotide standards to ensure that hydrolysis to the deoxynucleoside level was complete.

The [³H]BP-DNA adducts from bronchial tissues and synthetic adduct standards were co-chromatographed on a Varian model 5000 instrument programmed with a linear methanol in water gradient of 50 to 80% over 30 minutes on a Zorbax-ODS column (4.6 x 250 mm). A flow rate of 1 ml/min was maintained throughout the run and 0.5 ml fractions were collected with an ISCO model 1800 fraction collector fitted with a flow interruptor (ISCO model 590). The effluent was monitored at 330 nm with a Varian Vari-chrom UV detector and for fluorescence (Excitation = 330 nm; Emission > 389 nm) with a Schoeffel model GM 970 fluorescence detector. To each collected fraction, 5 ml of PCS (Amersham, Arlington Heights, Illinois) was added and all samples were counted in a LKB model 1216 liquid scintillation counter equipped with a DPM-PLOT package.

ANALYSIS OF BP METABOLITES IN CULTURE MEDIUM

HPLC analysis of benzo(a)pyrene metabolites released into the medium by cultured explants of bronchus was carried out on a LDC (Riviera Beach, Florida) gradient system driven by a LDC computer control module (CCM). A 4.6 x 150 mm ODS column (LDC 5 μ m Spherisorb) was eluted with 10 to 100% methanol in water gradients. The effluent was monitored for UV absorbance with a LDC Spectromonitor III detector and radioactivity with a Radiomatic Instruments (Miami, Florida) FLO-ONE detector. The tracings from these two detectors were recorded in real time and integrated by the CCM. Sixty fractions of 1 ml were collected in 7 ml mini-vials with an ISCO (Lincoln, Nebraska) model 1800 fraction collector fitted with an ISCO model 690 flow interrupter valve.

Radioactive material in the medium was analyzed by mixing 0.9 ml of media with 0.1 ml of methanol and injecting the mixture via a 1 ml sample loop onto the column. The fractions were mixed with 4 ml of PCS and the radioactivity was determined.

RESULTS

Prior to the initiation of these experiments, it was necessary to determine the period of time after death that bronchial tissues could be obtained and maintained as viable explants in culture. Therefore, using histologic techniques, the morphology of specimens of bronchus taken from 24 patients at autopsy was assessed prior to culture and after 1, 2, 3, 5, 7, 10, 14, 21 and 28 days of incubation. In addition, pieces of bronchus from each patient were placed in the center of 60-mm tissue culture dishes and incubated for 14 days to determine if epithelial cells would grow out from the periphery of the tissue onto the culture dishes (Stoner et al., 1980). Results from these experiments indicated that bronchial tissues obtained within 6 hours of death, could, in some cases, survive for 28 days in rocking explant culture, and exhibited epithelial cell outgrowths in primary culture. However, some variation with respect to these parameters was observed among patients. The morphology of bronchial explants prior to culture and maintained for 7 days in rocking organ culture are given in figures 1 and 2. Bronchial specimens collected from patients as long as 12 hours after death were usually non-viable.

The level of [3 H]BP covalently bound to the DNA of bronchial explants increased with time for a period of at least 24 hours (figure 3). In separate experiments, we observed that the levels of BP metabolites bound to bronchial DNA increased with the concentration of [3 H]BP in the medium - up to a dose of 10 μ M, the highest concentration used.



Figure 1. Specimen of human bronchus prior to culture. (X 200)



Figure 2. Explant of human bronchus maintained for 7 days in rocking organ culture. (X 200.) Note preservation of normal mucociliary epithelium.

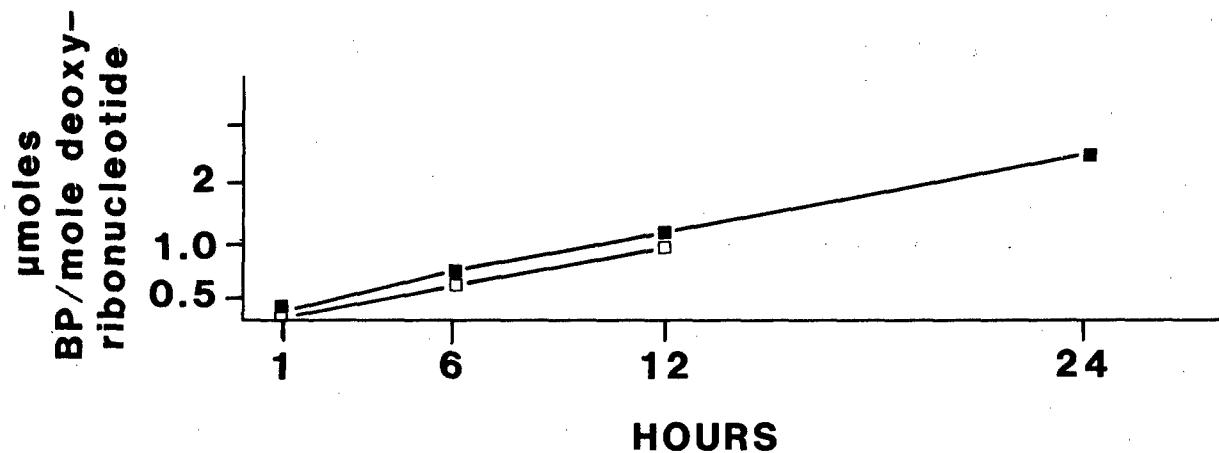


Figure 3. The level of $[^3\text{H}]$ BP-DNA binding as a function of time of exposure to $1 \mu\text{m}$ $[^3\text{H}]$ BP. Human bronchus (case no. 98 = \square ; case no. 100 = \blacksquare).

Figure 4 illustrates the variation among individuals in the levels of binding of BP metabolites to explant DNA in human bronchus. There was approximately a 10-fold variation in the levels of binding of BP metabolites to bronchial cell DNA. The mean level of BP metabolites bound to bronchial explant DNA was $3.1 \pm 1.9 \mu\text{mole BP/mole deoxynucleotide}$.

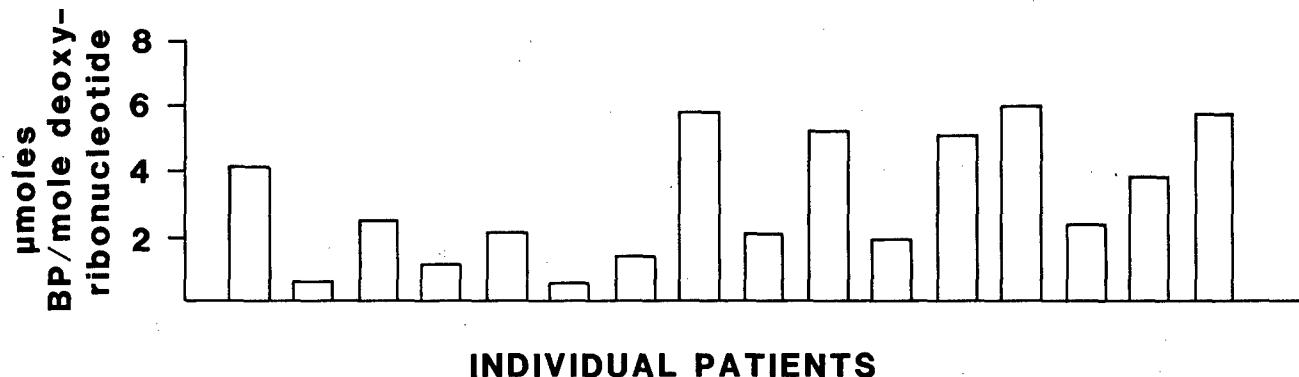


Figure 4. Graphic representation of the interindividual [^3H]BP-DNA binding levels in cultured explants of bronchus from the cases used in this study. All tissues were incubated for a period of 24 hours with 1 μM [^3H]BP.

The HPLC profiles of the BP-DNA adducts formed in bronchus explants after a 24 hour incubation with [^3H]BP are shown in figure 5. In both tissues, the majority of the radioactivity associated with the DNA co-chromatographed with the chemical standards (arrows, figure 5) previously shown to be adducts of the exocyclic amino group of deoxyguanosine (N^2) to the 10 carbon of the BP-diol-epoxide (Jeffrey et al., 1977). In all cases studied, 80% or more of the total radioactivity associated with the purified DNA was accounted for in the major adduct, 7R-BPDE-I-dG. A smaller peak eluting ahead of these two standards was consistently observed in the human bronchus (figure 5).

A representative HPLC chromatogram of whole medium is shown in figure 6. In all cases 2 major peaks of radioactive material were eluted: an early, polar peak (fractions 2-10) and a later peak (fractions 49-51). The commonly known metabolites of BP were eluted between these 2 peaks, as indicated on the chromatogram (figure 6). The early polar peak represented 35-83% of the total radioactivity in the medium from bronchial explants (Table 2). The second peak of radioactive material, which in all cases co-chromatographed with standard BP, represented 1-33% of the total in the medium from the bronchus. The commonly known metabolites of BP never represented more than 3% of the total amount applied to the column (data not shown).

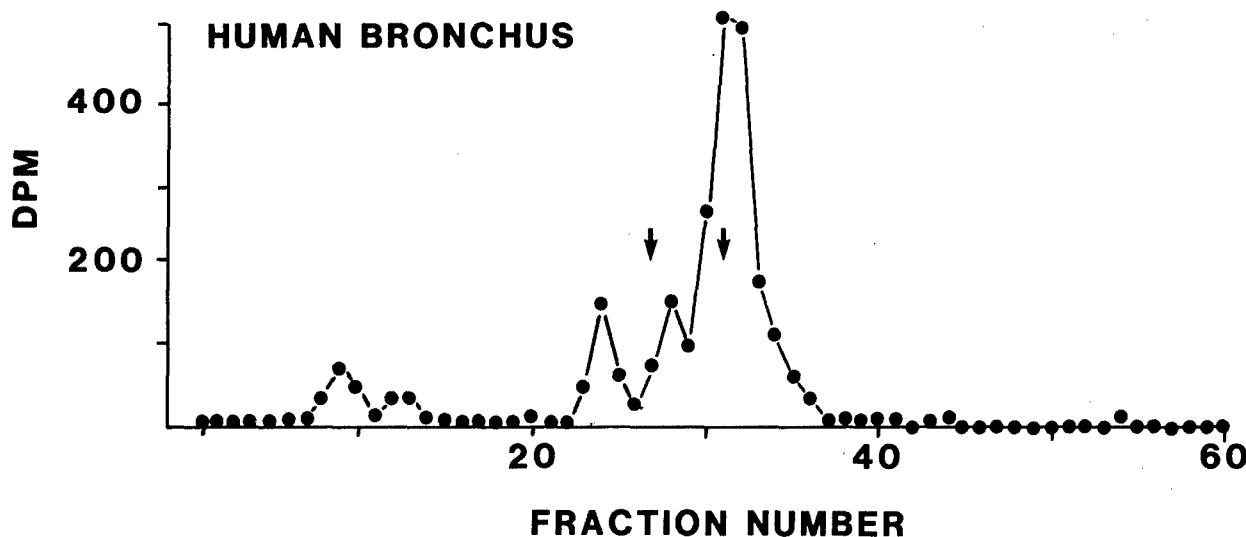


Figure 5. HPLC profiles (Zorbax-ODS) of $[^3\text{H}]$ BP-DNA adducts obtained from human bronchus following incubation with 1 μM $[^3\text{H}]$ BP. The arrows indicate the elution points of the two major adducts formed by the reaction of anti-BP diol-epoxide with calf thymus DNA, and represent 7S-BPDE-I-dG (peak fraction 27) and 7R-BPDE-I-dG (peak fraction 31).

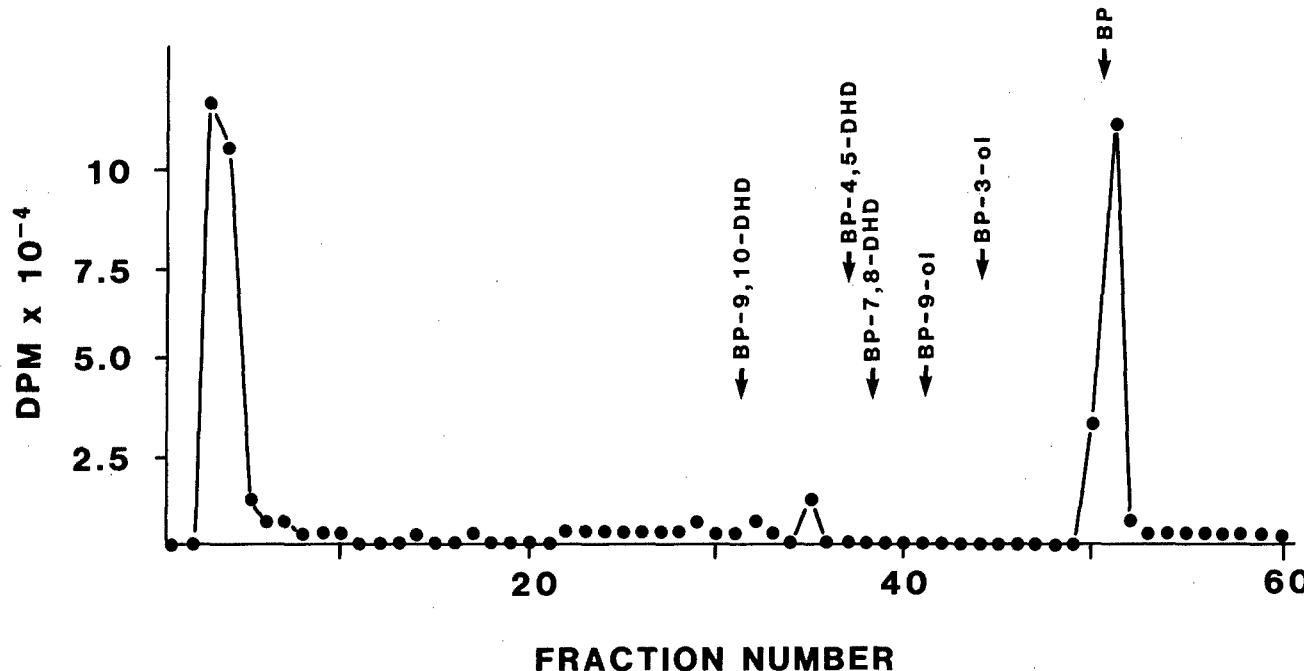


Figure 6. HPLC profile (Zorbax-ODS) of the media from human bronchus cultures (case no. 73) following 24 hour exposure to 1 μM $[^3\text{H}]$ BP. Arrows indicate the elution point of several benzo(a)pyrene metabolites.

TABLE 2. HPLC ANALYSIS OF RADIOACTIVE MATERIAL IN CULTURE MEDIUM FROM HUMAN BRONCHUS^a

<u>Case No.</u> ^b	<u>Percentage of Total Chromatographed</u>	
	<u>Polar Fraction</u>	<u>BP Fraction</u>
14	72.4	4.6
15	72.7	6.6
19	75.3	2.0
22	72.8	10.7
24	81.6	1.5
31	83.1	1.2
33	58.9	32.8
34	70.9	15.4
35	73.0	10.9
73	46.6	31.1
100	35.4	43.3

^a A 0.9 ml portion of the medium was mixed with 0.1 ml of methanol and chromatographed as described under Materials and Methods. Fractions were collected until the elution of [³H]BP was complete.

^b Medium from bronchial tissue of case no. 23 was not analyzed.

DISCUSSION

Epidemiologic studies indicate that lung cancer is more prevalent among cigarette smokers than among non-smokers. One of the many carcinogenic chemicals found in cigarette smoke is the polycyclic hydrocarbon, benzo(a)pyrene (Wynder and Hoffman, 1968). Therefore, the present study was undertaken to investigate the metabolism and DNA binding of BP in cultured explants of human bronchus.

Harris et al. (1976) and Autrup et al. (1978) investigated the metabolism of BP in human bronchial explants obtained from patients either at surgery or "immediate" autopsy. In the present study, all bronchial specimens were obtained at autopsy after periods of up to six hours following death. Our results confirm the reports of Harris et al. (1976) on the metabolism and DNA binding of BP by cultured human bronchus and demonstrate the feasibility of investigating the metabolism of at least one carcinogen, BP, in specimens taken at autopsy as long as six hours after death.

Explants of human bronchus obtained at autopsy were capable of metabolizing BP into forms that were bound to cell DNA. The average binding levels of BP metabolites to bronchial cell DNA

found in this study, [3.1 \pm 1.9 μ mole BP/mole deoxynucleotide (16 cases)] are similar to those found by Harris et al. (1976) who observed an average binding level of 1.2 \pm 1.2 μ mole BP/mole deoxynucleotide in bronchial explants cultured from 28 cases. Both studies employed similar conditions for explant culture of bronchus and time of exposure (24 hour) of the explants to [³H]BP. However, Harris et al. (1976) used a higher concentration of [³H]BP (1.5 μ M) in the medium and isolated the DNA by CsCl₂ density gradient centrifugation. It has been suggested that differences among individuals with respect to their ability to metabolize carcinogens into forms that bind to DNA could be associated with carcinogenic risk (Harris et al., 1976). In the present study, there was a 10-fold variation among individuals in the binding levels of BP metabolites to bronchial cell DNA (0.6 - 6.0 μ mole BP/mole deoxynucleotide). This is a smaller range of variation than that reported by Harris et al. (1976), who found a 75-fold variation in BP binding to bronchial DNA. The smaller variation observed in the present study may be due to the fact that all tissues were taken at autopsy whereas Harris et al. obtained tissues from both surgery and "immediate" autopsy. In addition, fewer cases were used in the present study. The maximum binding levels observed in the two studies were similar; in the present study, 6.0 micromoles BP/mole deoxynucleotide versus 4.9 micromoles BP/mole deoxynucleotide in the study of Harris et al. (1976). Histologic studies indicated that cultured bronchial specimens from some cases were more healthy than others. Therefore, it is likely that at least some of the interindividual variation in carcinogen-DNA binding levels may have been due to differences in cellular physiology and viability.

As reported previously by Jeffrey et al. (1977) for explants of human and bovine bronchus, Autrup et al. (1980) for human bronchus and rodent trachea, and Autrup et al. (1980) for explants of human colon, the major BP-DNA adduct observed in the present study for human bronchus co-chromatographed (figure 5) with one of the anti-BP diol-epoxide deoxyguanosine standards (7R-BPDE-1-dG). One of the two minor adducts co-chromatographed with the other anti-BP diol-epoxide deoxyguanosine standard (7S-BPDE-1-dG). The other minor adduct, eluting ahead of 7S-BPDE-1-dG, chromatographed in a manner similar to the "unknown II" adduct reported previously in human and animal respiratory tissues by Autrup et al. (1980). Osborne et al. (1981) demonstrated that reaction of anti-BP diol-epoxide with DNA produces minor, albeit unstable, adducts at the guanine O⁶ and N⁷ positions. On the other hand, one investigation has shown that BP-deoxycytidine adducts may elute in this region of the chromatogram (Meehan et al., 1977). There is no evidence of adducts to deoxyadenosine in human bronchus since they would be expected to elute after the deoxyguanosine adducts (Autrup et al., 1980).

An unexpected result was the observation that the majority of the radioactive material in the medium following incubation of human bronchus explants with [³H]BP was either unmetabolized BP or present in a highly polar fraction, and that very little (~3%) of the total amount chromatographed eluted with the commonly known unconjugated metabolites of BP (figure 6). In order to quantitate these, it was necessary to pool media and prepare an ethyl acetate extract. These studies are now in progress. The amount of radioactivity in the highly polar fraction was inversely correlated with the quantity of unmetabolized BP. Preliminary results have also revealed that, in most cases, the majority of this polar material cannot be extracted with organic solvents and is refractory to hydrolysis by β -glucuronidase and aryl sulfatase, possibly indicating the presence of glutathione conjugates or other conjugated or unconjugated polar material. It will be of interest to determine if this highly polar material is similar to that demonstrated by Boroujerdi et al. (1981) in the pre-9,10-dihydrodiol region of their HPLC chromatograms of rat biliary excretions following i.v. injection of [³G-H]BP.

Similar to the DNA-binding data (figure 4), large interindividual differences exist in the extent of BP metabolism (Table 2). Part of this variation is undoubtedly due to differences in the amount of tissue incubated per unit volume of medium and, to some extent, the relative viability of the explants.

In summary, explants of human bronchus were fully capable of metabolizing the carcinogen, BP, into forms that were bound covalently to cellular DNA. When compared to other cultured human tissues, the bronchus appears to have a greater ability to metabolize BP into forms that bind to DNA than any other human tissue yet examined. In an earlier report, Autrup et al. (1980) summarized comparative studies on BP metabolism in explants of different human tissues. On the basis of this summary, and the results of the present study, we may rank human tissue with respect to its ability to bind metabolized BP as follows: bronchus > trachea = esophagus > colon.

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OPEN FORUM

DR. STONER (Medical College of Ohio): We'd like to begin the Open Forum for discussion of the papers presented on Animal Modeling of Lung Carcinogenesis. I would first like to ask our chairman, Dr. Kiesow, to summarize some observations he made of the talks this morning and then we'll start the open discussion.

DR. KIESOW (Naval Medical Research Institute): There were several points made in this morning's presentation that struck me as scientifically interesting and at the same time real advances in the field of toxicology with significant potential for future toxicologic research. In the interesting talk on the effects of asbestos fibers Dr. Mossman described the effects from differences in chemical structure or composition. In recent years the effect of distribution of chemical charge in the crystal lattice of the inhaled material has been reported to be significant, apparently in conjunction with the control and stimulation of cell growth. These are significant to the effect that external electrical fields seem to have influence on controlling the rate of cell growth and changes in charges in the crystal lattice may be the mediator for this. I wonder if Dr. Mossman may have some comments as to what effect the potential differences in crystal structure in these various asbestos compounds may have both as direct stimulator or mediator of growth and secondly upon the binding of the carcinogenic compound. Most striking in the discussion was the apparent ease with which toxicologists nowadays grow organs and tissues in culture, particularly epithelial tissue. I do recall that some fifteen years ago that was an art and very difficult to do. But nowadays it seems that there is a great potential for research resulting from this capability to culture various tissues, and I would be most pleased to have these speakers elaborate a little more on the nitty-gritty factors which constitute the advances and which usually are never mentioned in published papers. It comes to mind that James Clark Maxwell decades ago accused Amperé, while he was a great physicist, of being also a gentleman who was extremely capable at destroying all the scaffolding that he needed to reach his level of discovery so nobody could recognize the steps necessary to repeat his experiments. It seems to me that there may be some of the scaffolding here in those talks which would be very interesting for those of us who have an interest in using these techniques. It is particularly intriguing to learn that tissue cultures and also cell lines have become available for use in the testing of carcinogenic substances. If indeed cell lines are becoming acceptable substitutes for animals in the study of tumorigenesis, I think that would be a great advance. In particular, it would be useful if these cell lines or primary cultures could be grown from tissues of different origin from different species, and therefore, have hopefully retained some if not all of the characteristics of metabolic pathways and also metabolic rates that these tissues originally had. We should recognize that many of the toxic effects are not equally distributed among different

cells and different organisms as well. So to learn more about the toxicity of chemicals for man I think that the tissue culture method is probably extremely useful and it would be helpful to learn more about it.

DR. MOSSMAN (The University of Vermont): You asked about the effect of electrical charge with regard to asbestos. Approximately two years ago some data from a group at UCLA were published in Nature and aspects of the same study were published in Science in which the investigators used hemolysis of the red blood cell as a quantitative measure of damage due to asbestos particle charge. They found that a cytotoxic type of asbestos would cause release of hemoglobin from the red blood cell which could then be measured spectrophotometrically. They looked at chrysotile or white asbestos which has a strong positive charge that is supposedly due to the high content of the magnesium ion in the asbestos fiber. In contrast, they also looked at crocidolite which is a negatively charged type of asbestos and found that cytotoxicity was directly related to the positive charge of the chrysotile. They ran some experiments in which they leached magnesium from the chrysotile fiber thus leaving it with a negative charge and showed that cytotoxicity was ameliorated as the chrysotile took on a negative charge and vice versa with the crocidolite. So it certainly appears that the charge of asbestos is important in cytotoxicity. This does not appear to be the case regarding cell growth and proliferation. In our system, for example, we have observed the induction of squamous cell metaplasia and basal cell hyperplasia with negatively charged asbestos forms such as crocidolite and amosite. Chrysotile asbestos, on the other hand, does not produce this prolonged growth proliferative effect on the epithelium, so at this time we cannot equate cytotoxicity directly with growth and proliferation of these cells. There has been one experiment reported on the effect of asbestos charge on tumor induction which was done by Morgan's group in England. They leached chrysotile asbestos, the positively charged asbestos, and removed magnesium from the fiber which is the only way to take the positive charge off the fiber but, unfortunately, when you do this you also alter the crystallographic structure of the asbestos. With regard to mesothelioma, in their test system they injected the asbestos intrathoracically into the animals and watched for the development of mesothelioma. The numbers of tumors induced were so small that they found no statistical difference between the positively charged asbestos and the negative.

I believe the second question related to the charge effects with regard to binding of the polycyclic hydrocarbon and since a polycyclic hydrocarbon is an uncharged, really a lipophilic, type of substance, I should emphasize that there is really no binding and there is no charge interaction. It's more an adsorption phenomenon of the asbestos particle by polycyclic hydrocarbon asbestos. We've done comparative studies looking at chrysotile which is a pliable curly type fiber and crocidolite, a rod-like fiber,

and found that on a weight basis chrysotile binds more of the polycyclic hydrocarbon. We believe that this is a function of surface area because chrysotile is comprised of many fibriles which all could act as adsorbents of the polycyclics.

DR. STONER (Medical College of Ohio): Thank you, Dr. Mossman. Dr. Kiesow also asked about the technical tricks used for the culturing of epithelial cells. Would you elaborate somewhat on that, Dr. Steele?

DR. STEELE (National Institute of Environmental Health Sciences): I won't take credit for all of the tricks, but some of the things that have made possible the culture of epithelial cells in the last fifteen years is some diligent work by cell biologists testing different kinds of media, different hormones which can now be isolated, different new kinds of nutrient additives, and new ways of culturing epithelial cells in the midst of fibroblast contamination. The latter is one of the nitty-gritty things that people don't like to talk about when they isolate epithelial cells. With the organ culture system that I described in my presentation, the fibroblasts can be removed by selective trypsinization since fibroblasts do not attach to culture dishes as tightly as do epithelial cells. The fibroblasts can also be removed by mechanical scraping and this involves long hours physically removing fibroblasts under the dissecting scope. But this was necessary since in the earlier medium we were using the fibroblasts that grew much faster than the epithelial cells and eventually we could cover them and cut off their nutrient supplies. With the primary cultures I described, we used a media that Dr. Wu developed consisting of very low concentrations of serum. We've found that high serum concentrations are particularly toxic to the tracheal epithelial cells, and we were able to develop a hormone supplemented medium that allowed us to lower the serum concentration. This new medium not only allows the maintenance of long-term primary epithelial cultures, but the low serum content inhibits fibroblast growth. Another thing that you have to do in an isolation procedure is to try a number of different enzymes, proteases, collagenases, a variety of enzymes to find that enzyme which will give you the cleanest cell preparation. After six or eight months of trying enzymes, we settled upon a protease type-6 that worked fine but unfortunately is no longer commercially available. It isolated epithelial cells and we would only find fibroblast contamination in about one percent of our culture dishes. We are now in the process of going through this search again trying to find a suitable enzyme preparation which will give us pure isolation of epithelial cells.

Cell lines are much easier to grow than primary cell cultures. They'll grow on a much simpler medium if you can derive epithelial cell lines. We use a serum-free system to grow non-transformed and transformed cell lines to look at hormonal dependence of these. One of the tricks you have to use for primary cell culture for rat tracheal fibroblasts is collagen coding. In order to keep the

cells around for a long time you must have them collagen coded. You can use collagen gels or collagen sponges like Dr. Stoner does. Once the cell lines make this change, they do not require substrate. The cell lines also don't require the use of fibroblast conditioned media.

One of the drawbacks to culture systems that we still encounter is that the cells tend to become undifferentiated with time. This is one reason why some studies require organ cultures in which you maintain cellular differentiation. You are limited somewhat by the fact that you can't study very early cellular changes after a chemical exposure with organ cultures other than by sacrificing the tissue and sectioning it. With cell cultures, you can make a number of measurements right after exposure without sacrificing that culture and look at the ultimate outcome.

DR. STONER: I can make some additional comments along these lines, in terms of the explant cultures of human tissue and also about some of the animal tissue models that we've been working with. The key factor that's been helpful in maintaining these tissues for relatively long periods in culture is to provide a condition where the tissues are exposed alternately to the culture medium and to an elevated concentration of oxygen in the atmosphere in the rocking organ culture system. Generally, we've gotten much better survival of all the tissues that we've worked with so far in the rocking organ culture system than we have in stationary culture. It really doesn't make a lot of difference what medium you use and one can get by without utilizing serum. For cell culture we found that the method used to dissociate cells prior to their subculture is very important with epithelial cells, preferably using very small amounts of enzyme or no enzyme at all, thereby improving subculture of these cells. We use a mixture containing 1% polyvinylpyrrolidone, 1% EGTA, and 0.02% trypsin. A small amount of trypsin helps dissociate the cells but is not enough to be toxic. We find that this is our best method for being able to dissociate the cells and subculture them. If we use 0.2% or higher trypsin solutions commonly used for dissociating tissues, we find that these are generally very deleterious for our epithelial cells in culture.

Another comment I want to make is that temperature seems to be a critical factor for the esophageal epithelial cells we've been working with and also for epidermal cells. It would appear that the differentiation of these cells is retarded somewhat by lowering the temperature at which the cells are being cultured. One can maintain a primary outgrowth from pieces of tissue significantly longer at 31°C than at 37°C. Finally, with respect to serum, I think many people are finding now that if one can use a medium supplemented with some appropriate growth factors such as epidermal growth factor, hydrocortisone or insulin, one can eventually derive a medium for themselves in which no serum is included at all. This has been done with human bronchial epithelial cells and when serum

was added back to that system, it was actually shown to be toxic for the cells. Many commercial lots of serum are quite toxic for cells in culture. We've also obtained similar results with rat esophageal epithelial cells by tailoring the medium constituents until we can grow these cells in the absence of serum and we've found that adding back serum in concentrations as low as 2% can actually decrease or inhibit growth of these cells. Eventually it will be possible for many epithelial cell types to be grown in serum-free medium when appropriate additives are used and the proportions of constituents present in the medium are suitable.

DR. MOSSMAN: I'd like to add to the discussion of organ culture protocols. There has been a fair amount of work done in all of our labs that has shown the medium constituents can alter the differentiation of the organ culture over periods of time. Depending upon the objectives of experiments, the choice of medium is very important. For example, if we are trying to establish a condition where cell proliferation is encouraged as a means of transforming the tissues, we would want to stick with a complex medium full of growth factors. This type of medium without the addition of Vitamin A will cause squamous metaplasia and basal cell hyperplasia over a period of time which can be documented by uptake of tritiated thymidine. On the other hand, if you're interested in looking at the effects of an agent which you feel might be toxic to epithelium and want to maintain a normal differentiation of either hamster or rat tissue, you would want to use a minimum essential medium containing Vitamin A which maintains the mucociliary characteristics of the epithelium. That is really an important consideration.

DR. CAVENDER (The MITRE Corporation): I'd like to ask Dr. Mossman a question related to some studies that I was involved in where we exposed animals to chrysotile asbestos. We found that very little material was ever deposited in the tracheal region of the animals. Most of the fibers were deposited in the lower regions of the bronchioles and the very fine short fibrous material was cleared very quickly, but the longer fibers stayed and these entered the lung through the interstitium. How would you relate the finding in a tracheal organ culture system to what one expects in a respiring lung?

DR. MOSSMAN: I think your studies support what we've observed, that there is asbestos uptake by the epithelium, be it the alveolar or the bronchiolar or the tracheal-bronchial epithelium. There was, although in limited amounts, some uptake of asbestos particles by the bronchial epithelium in your studies. You used chrysotile asbestos and I would assume that the deposition of that is going to be different than with crocidolite which is the asbestos form that I've done most of my work with. We still have to deal with the evidence that the tumors caused by exposure to asbestos are for the most part bronchogenic carcinomas and not peripheral tumors. Studies by Lippman at New York University with

crocidolite have shown that there is increased retention of the fiber at the bronchial bifurcation with this type of asbestos. This was an inhalation experiment with radioactively tagged material. I would say that your studies were supportive of what I've found as far as a quantitative difference in uptake between alveolar versus trachea-bronchial epithelium. I think that this is due not only to the different size characteristics of the asbestos but also to the different types of asbestos that were used.

DR. CROCKER (University of California, Irvine): Dr. Stoner, when you were ranking carcinogens on a micromoles per kilogram basis where they were productive of alveolar adenomas in the strain A mouse model, did you ever rank them in terms of their LD₅₀ or their toxic potential for other organs or their carcinogenic potential for normal target organs? For example, in some cases the normal target organ is not the lung, but incidentally you also found adenomas. It would be nice if they could be ranked in terms of these other biologic properties for which they may have a higher potential effectiveness than they do for adenomas.

DR. STONER: We haven't done that kind of ranking, but that's a good idea. One thing we know about this system is that there has been very little work done on the pharmacokinetics of distribution of carcinogens or on the metabolism of carcinogens in any of the organs in the Strain A mouse. I think those kinds of experiments need to be done so we can understand something about the differences in susceptibility of the different tissues such as lung versus liver in this system. We are beginning to conduct studies in pharmacokinetics with some of the nitrotoluene compounds and we're going to do it also with some of the known liver carcinogens and lung carcinogens. The test compounds have generally been administered intraperitoneally in most of the assays with the strain A mouse and there's really very little data regarding the comparison of the route of administration on the tumor response. We're comparing the ip versus the oral route of administration of compounds and we're beginning to find some very striking differences.

DR. CROCKER: Dr. Steele, while your own work has been largely with in vitro studies, you are part of a group that has also been doing some organ, especially tracheal, grafts and I believe that in that system you've examined the effects of asbestos and other fibers. Is that true?

DR. STEELE: That's true. We have a heterotopic tracheal transplant in which my colleagues looked at promotion of dinitrobenzanthracine carcinogenesis. In the graft they used 200 microgram DNBA pellets inserted inside tracheas which were then tied off and transplanted subcutaneously into host animals. Following a two-week exposure, the DNBA pellets were removed and replaced with gelatin pellets containing, I believe, 100 micrograms asbestos. In these studies they saw a five-fold enhancement in the tumor response if DNBA exposure was followed by asbestos treatment compared with

DNBA alone. This level of asbestos alone produced an inflammatory response in tracheal grafts but no tumors were found. With initiation alone, they could find various dysplastic and metaplastic lesions after two to four months but very few of these lesions actually progressed to become tumors. What they wanted to demonstrate was a promotional function of asbestos in the induction of tumors unrelated to its carrier function of getting carcinogens to the cells.

DR. CROCKER: I talked with Dr. Mossman about the degree to which she was able to use clean asbestos fibers to remove absorbed materials including polycyclic hydrocarbons for an assay, to establish that the asbestos was in fact a promoter rather than an initiator which Dr. Steele's statements would suggest. I would like to ask her a further question. Dr. Mossman, have you done any *in vivo/in vitro* comparisons of asbestos response? Have you compared intratracheally instilled asbestos in living animals versus the effects of asbestos in tracheal grafts?

DR. MOSSMAN: No, I have not intratracheally instilled the material for comparison with grafts. I have used the heterotopic tracheal graft as Dr. Steele has described and a problem with that system was an inflammatory response. Our preliminary observations led us to believe that in some cases this inhibited or arrested tumor progression, and that is why we use the organ culture system. When the tissue goes into the host in culture, the asbestos is inside the cell as opposed to in the external environment and one bypasses defense mechanisms and doesn't get an immunologic response. I would say that the experiments by Topping and Nettisheim described by Dr. Steele are supportive of what we're observing in our *in vitro* system, that the properties of asbestos are very similar to a classical tumor promotor. Thusfar we've looked at stimulation of cell membrane enzymes, stimulation of polyamine synthesis, stimulation of cellular division, and inhibition of differentiation.

DR. MUKERJEE (U.S. Environmental Protection Agency): I have a question for Dr. Mossman on a similar line. Gabriel has found in the mouse that asbestos can cause tumors similar to those seen in the *in vivo* system. You say that this effect is a promotion action by asbestos. In the EPA, we have listed asbestos as a carcinogen based on the data from Selikov as well as Nicholson's data and also using Gabriel's work in the mouse. If it's only a promoter we have a problem. Is there any *in vivo* data which can be utilized to demonstrate that asbestos is a promoter rather than a direct carcinogen?

DR. MOSSMAN: Yes, in fact, most of the studies that have been done with either intratracheal instillation or inhalation of asbestos have given negative results unless a polycyclic hydrocarbon is either administered at the same time as the asbestos or on the surface of the fiber. I'm aware of some of the studies that have been done with asbestos alone that have given neoplasms, but in general

these are not bronchogenic neoplasms. They are mesotheliomas and in that case the picture is entirely different from bronchogenic carcinoma. One problem in interpretation of a lot of the inhalation experiments is that native asbestos, including the UICC reference sample, is contaminated with small amounts of polycyclic hydrocarbons. This was what I believe prompted Dr. Crocker's questions. It is essential before you do any of these studies to extract the organic contaminants from the asbestos fiber.

DR. STONER: I would like to say that I think it's a bit premature to assume that asbestos might not be a mutagenic agent inasmuch as we've done some studies on the penetration of amosite asbestos fibers in cultured human bronchial epithelial cells using high voltage electron microscopy and it has been possible to see the fibers penetrating to the cytoplasm and to the nucleus of these cells. It may be that there is some sort of DNA damage that perhaps has not been picked up in similar conventional mutagenicity assays that asbestos might do to cells. Asbestos could actually be a genotoxic carcinogen but our assays simply aren't adequately sensitive to prove it.

DR. MOSSMAN: I should emphasize that in the Ames assay asbestos is not mutagenic; however, there have been studies showing chromosomal aberrations in various mammalian cell types in culture. We've looked to see whether there is single strand breakage of DNA in asbestos exposed tracheal epithelial cells and have not been able to document any. Again, it could be the insensitivity of the system and I think at this point I wouldn't say that asbestos is not carcinogenic. I will say that it's probably a weak or non-initiating substance and more of a promoter-like compound.

DR. PEPELKO (U.S. Environmental Protection Agency): Dr. Kouri, have you ever done any work where you adsorb your carcinogens on inert carbon particles?

DR. KOURI (Microbiological Associates): No, we've not used carbon particles, we've used ferric oxide and have had no real effect, at least in this particular model system. The hamster model system that Dr. Mossman was talking about is influenced in vivo by addition of benzo(a)pyrene or methylcholanthrene onto a ferric oxide molecule. We had no measurable effect in our model system. The problem with carbon is that the compounds stick to it and you can't get it off. There's actually a question of semantics in terms of promotion and co-carcinogenesis. In my definition, they are not the same thing. Asbestos can likely be a co-carcinogen; that is, it can be a sink for certain types of chemicals and allow either an easier transport or cause more efficient induction of enzyme just by staying around a little bit longer and providing a more biologically effective dose of the particular chemical to the particular cell in question. In that regard none of us would call that an initiator function although it's actually aiding in the initiation process per se. On the other hand, it may also contain

promotion type activity and that is the ability to cause proliferation after the addition of the chemical carcinogen. So, likely asbestos can have more than one effect and most likely does.

DR. COURI (Ohio State University School of Medicine): This morning I learned that strain A mice have something like 70% or 100% tumor incidence in a couple of years and yet the C57BL6 or the C57BL6/CH3F1 have almost zero or very low incidence of lung tumors. How does one choose, in the design of an experiment to study lung cancer, the strain of mouse to use between the one that gives 100% tumor background or the one that gives me zero incidence?

DR. STONER (Medical College of Ohio): Well, one of the reasons that some chose to use the strain A mouse (and actually it was chosen long before I began work in this area) is that it's very sensitive to the induction of tumors with carcinogens and it's the sensitivity of that assay that made it attractive. During a six-month bioassay, the spontaneous occurrence of tumors in the strain A mouse is about 0.2 to 0.3 tumors per animal. That means that probably one out of five or one out of four animals actually will develop a single tumor per lung. It's true that in older animals kept for a year and half to two years virtually all of them will spontaneously develop approximately one tumor per lung. We have a fairly low tumor incidence background during the time at which this assay is being conducted. If you have a carcinogen that induces three to four or five or twenty or even as high as 100 tumors per lung, such as one can get with a 2 milligram dose of methylcholanthrene, there's a very significant difference there. One of the criticisms of this assay is that alveolargenic lung tumors are not very prevalent in the human population. Most human lung cancers occur in the epithelium of the bronchus or the terminal bronchioles and not in the alveolar epithelium. While that criticism is valid, what we're asking of this system is essentially will it detect carcinogens? And if it will do that and will not pick up non-carcinogens, then we're happy. But the question of saying that if a compound is positive in this system for alveolargenic tumors it will be active for bronchogenic tumors in humans, we really don't know. Certainly it is a reasonable assay for detecting carcinogens. Dr. Kouri's system is really more relevant to human cancer.

DR. COURI: When you use the term sensitive, I take it you mean that it just causes tumors in that animal and that's without consideration for dose required to cause the tumor. It seemed to me that some of the doses of tumor causing agents in your slides were quite high to result in tumor, even in strain A mice. Is that correct? I assume that by sensitivity you mean that it is positive for tumors without regard for dose effect.

DR. STONER (Medical College of Ohio): If I examine the doses of methylcholanthrene necessary to give a positive carcinogenic

response in this system, we could easily get that with 50 micrograms, somewhere in that range. Much lower than a milligram dose. A one milligram dose will give us an average of 40 to 50 tumors per lung, whereas a measurable positive response in the strain A mouse over six month's period of time would be somewhere in the range of 0.8 to 1 tumor per lung. We can use a considerably lower dose than that I discussed.

DR. KOURI (Microbiological Associates): I agree with Dr. Stoner. The two systems are generated and derived for two different reasons. The strain A adenoma model system has been used to screen for those particular chemicals that may prove to be biologically active and at least in that model system they may prove to be biologically active in humans. In that regard, it was derived specifically for that purpose. It's fairly rapid and has a fairly well defined visible endpoint. I don't like the endpoint because like the skin model system with papillomas as an endpoint, they're benign tumors. I doubt if you can really address the question of mechanism with the adenoma model system. It performs a particular purpose in terms of being able to screen biologically active compounds. The strain A mouse has a fairly high capacity to metabolize chemical carcinogens and lung AHH enzymes are easily induced. I know that there are some problems now that Dr. Stoner might like to address about strain variations, substrain variations like A/Strong versus A/JAX versus some other ones and I wonder if there is any biochemical or biological basis for those differences. The model system that I use was developed to try to address the question of carcinogenesis mechanism and to generate a relevant tumor. A tumor relevant to the human situation that would grow, metastasize and act at least similarly to what we think the same sort of tumor would do in humans. With that point of view we want to have a model that has a very low spontaneous incidence of neoplastic disease because we want to be able to discriminate as best we can between any chemically induced phenomenon and this naturally occurring tumor incidence. Even under those conditions, in tobacco smoke the smoke studies that we are conducting in order to have sufficient numbers for valid statistics, we have 2,000 animals exposed by nose only inhalation of smoke exposure, we have another 1,000 animals as sham exposed controls and other groups of 700 animals exposed to smoke plus benzo(a)pyrene smoke plus methylcholanthrene. It's not a small study. Even under those conditions we are trying to reproduce in animals tumors seen in man induced by smoking three packs of cigarettes a day. In man smoking that much, only one out of every twelve individuals come down with lung cancer. We expect in a mouse we may be able to get a little bit higher incidence than that, but we're trying to discriminate differences of only about 10% variation among the groups. So far we haven't done it. As much as I think the model system seems to be relevant, it hasn't yet picked anything up in cigarette smoke. Now we have a question about whether we cleaned up the mouse too well, it has no virus infection, the air is filtered, the animals are very clean and they live to a fairly long age. There is still some

constant stress associated with exposing animals to the smoke. It doesn't allow them to grow as rapidly and they don't get as fat as the controls. Our control animals are actually dying faster than our sham or smoke exposed animals, perhaps because they are more obese than the sham or exposed animals. They are also showing a variety of lesions that the sham and smoke exposed animals aren't showing.

Our system and the strain A mouse system are really two separate model systems trying to describe different things. I do think our model system has relevance to trying to understand what causes carcinomas, the biological expression of those carcinomas, how they grow, when they grow, how they eventually kill an animal.

DR. ALI (Ohio State University School of Medicine): Dr. Kouri, how early can you pick up the preneoplastic changes that you mentioned with the squamous cell carcinoma and adenocarcinoma? You mentioned that your animal dies earlier with a squamous cell carcinoma because of the potential behavior of these tumors probably due to metastasis to the heart, myocardium and the kidney. Is this the primary cause of death or mainly due to the invasion of the squamous cell carcinoma of the lung or to the metastasis to the heart or the kidney?

DR. KOURI (Microbiological Associates): We find squamous neoplasms which look like a carcinoma as early as eight weeks in those particular studies I discussed this morning. We've had squamous cell carcinomas with metastasis as early as ten weeks. That would be after the fifth intratracheal inoculation. That means they were actually developing carcinomas at the time at which the chemical was being administered. Early alveolargenic lesions are a little more easy to describe because they have a longer latency period. We find alveolar hyperplasia within two weeks after chemical treatment. The hyperplasia seems to progress to what we call a noncompressing alveolar nodule; it looks like a small adenoma. Then it becomes a compressing nodule in which the surrounding parenchyma is actually compressed. That occurs between the 20th and 30th week and then we find some alveolar adenocarcinomas by the 40th and 50th week. We find higher and higher incidence of adenocarcinomas from the 50th week on after treatment. Preneoplastic changes are found fairly quickly after chemical treatment.

Your second question was about squamous cell carcinomas and the cause of death in these animals. I think I agree with what you said. I don't know if I said anything that was different, but I believe the squamous cell carcinomas are in fact growing invasively right along the pulmonary vein directly to the heart and are causing cardiac infarcts and cardiac insufficiencies that kill the animal. I think the cause of death is the tumor producing either left atrial obstruction, renal infarction, or pulmonary insufficiency.

DR. STONER: I would like to comment on Dr. Kouri's question about the differences in the responses of the A sub-strains to urethane. We obtained five breeding pairs of A/Strong mice about a year and a half ago and tested their response to urethane and found responses very untypical of what we would expect and, however, with A/JAX mice and A/Heston mice, the responses were about as they had been historically with the production of about one tumor per milligram dose. As it turns out, the A/Strong strain is no longer a pure A strain. It has been inadvertently mixed in with another strain of albino mice which is much less susceptible to lung tumor induction with carcinogens. If you're planning on using the strain A system, I would highly recommend you avoid using the A/Strong substrain.

DR. KOURI (Microbiological Associates): The A/JAX strain is satisfactory?

DR. STONER: Yes, it is.

MR. KOTUR (Battelle Memorial Institute): Dr. Stoner, would you elaborate on the age of human donors of tissues for your in vitro work?

DR. STONER: The age of human donors of tissue for in vitro work varies considerably from very young children up to as much as 89 or 90 years old that I believe we have obtained tissue from. It doesn't seem to make much difference what age the patient was from which the tissue was obtained for culture as explants. In terms of initiating cell cultures from explants, we find that the outgrowths of epithelial cells from tissues taken from very young patients are significantly better, more luxuriant than outgrowths from tissues taken from older patients.

DR. MacEWEN (University of California, Irvine): Dr. Stoner, you demonstrated a rather large variation in benzo(a)pyrene metabolism in tissues from individual donors. Could those differences have been related to age or perhaps the sex of the donor?

DR. STONER: Based on the few numbers of specimens that we've had, it's difficult to be conclusive about this point, but it does not seem to be related to either age or sex.

DR. MacEWEN: What about smoking status?

DR. STONER: Yes, those individuals who had cancer from which we obtained tissue were for the most part smokers and they had a better ability to metabolize benzo(a)pyrene than tissues taken from patients who were non-smokers.

SESSION II

THE IMPORTANCE OF SEX-SPECIFIC EFFECTS IN TOXICOLOGY

Chairman

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**ORGANIZATIONAL EFFECTS OF HORMONES AND HORMONALLY-ACTIVE
XENOBIOTICS ON POSTNATAL DEVELOPMENT**

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At a very early stage of development the male and female are morphologically indistinguishable. Chromosomal sex established at the time of conception directs the development of either ovaries or testes that are responsible for phenotypic expression. The secretory activity of the fetal testes is responsible for the transformation of the indifferent urogenital tract and external genitalia into the male phenotype. Similar relationships are involved in the sexual differentiation of the brain. Although the effects of hormones on the adult brain are normally reversible, hormones are capable of causing permanent effects during an early critical period of development that result in sex differences in neuroendocrine secretion, behavior, and reproduction.

Hormone stimulations of lordosis behavior, ovulation and enzyme activity are reversible responses which can be termed "activational effects" and reflect direct hormone action which require the presence of the effector. Activational effects may be contrasted to the developmental or "organizational effects" of steroids on the reproductive tract and brain which occur during a limited critical period of fetal or early postnatal development and are manifested in adults. Expression of the latter type of hormone regulation may not occur until after the onset of sexual maturation and has also been defined as imprinting or programming. In order for these organizational effects to take place,

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hormone action must occur during a sensitive period of early brain development. In humans this is primarily during the last trimester of pregnancy, while in laboratory rats the critical period is primarily during the first week after birth, when the testes of the male secrete testosterone (Dobbing and Sands, 1979; MacLusky and Naftolin, 1981; Ehrhardt and Meyer-Bahlburg, 1981).

There is sufficient evidence to suggest that sexual differentiation of the reproductive tract, sexual behavior, and endocrine secretion in humans and lower species is dependent on organizational effects. McEwen (1976) describes a mechanism for imprinting in the developing rat in which testicular androgen reaches the brain and is aromatized to estrogen (Reddy et al., 1974; Naftolin et al., 1975; McEwen et al., 1977); this estrogen binds the estrogen receptor (McEwen, 1977) and the estrogen receptor-ligand complex is translocated to the nucleus where transcription, translation, and post-translational processes take place to program for a male type of endocrine secretion and sexual behavior. These sex differentiated parameters are mediated via the hypothalamic-pituitary-gonadal axis.

The fetus and newborn are particularly susceptible to hormonal imbalance as a consequence of genetic endocrine disorders, abnormal pregnancies, drug treatment, or exposure to chemicals or factors that can change the hormonal milieu during the critical period of development. Patients with congenital adrenal hyperplasia synthesize low concentrations of cortisone but high concentrations of adrenal androgen, resulting in masculinization of the external genitalia of genetic females. These prenatally androgenized females have been reported to display intense active outdoor play, increased association with male peers, high energy expenditure (tomboys), decreased parenting rehearsal such as doll play and baby care, and a low interest in the role rehearsal of wife and mother versus having a career (Ehrhardt et al., 1968; Ehrhardt and Baker, 1974). The human clinical syndrome of testicular feminization of genetic males is characterized by tissue insensitivity to androgens, appearance of female external genitalia in the presence of testicular gonads, and normal testosterone production (Masica et al., 1971; Money et al., 1968). Sex hormones have been used clinically in the treatment of high risk pregnancies. It has been found that prenatal exposure to exogenous progesterone correlates negatively with physical activity level in childhood and also with heterosexual activity in adolescence (Meyer-Bahlburg et al., 1977; Ehrhardt et al., 1977). In girls there was negative correlation of tomboyism and positive correlation of traditional feminine activities in childhood. Prenatal treatment with medroxyprogesterone acetate has also been found to feminize psychosexual parameters. Prenatal exposure to estrogens has been reported to interfere with normal masculinization in the male offspring (Yallow et al., 1973).

In utero exposure to diethylstilbestrol (a nonsteroidal estrogen) plus progesterone has been reported to affect neurobehavioral and reproductive capabilities of male offspring (Bibbo et al., 1975). Diethylstilbestrol has also been shown to be a transplacental toxin that can result in vaginal adenocarcinoma in post-pubertal females prenatally exposed to this compound (Herbst et al., 1971).

In addition to obvious sex differences in physical, reproductive, endocrine and behavioral correlates, a number of studies have demonstrated sex differences in biochemical metabolism. There is evidence that sex differences influence the rate of disposition of chlordiazepoxide (Greenblatt et al., 1977; Roberts et al., 1979) and diazepam (MacLeod et al., 1979) in man. These differences may be due to sex differences in steroid metabolism. Fishman et al. (1980) using radiolabeled estradiol showed major sex differences in estradiol metabolism in humans. Men excreted less estradiol and its metabolic products in urine than women, and of that fraction metabolized in men, less was transformed via 2-hydroxylation than in women. Pfaffenberger and Horning (1977) looked at urinary steroid profiles by gas chromatography and found two different types of steroid metabolism for premenopausal females, one group displayed metabolic profiles closely resembling those of males. The authors suggest that these sex differences may be due to late fetal or neonatal hormonal effects (Pfaffenberger and Horning, 1977). Men have been reported to have higher plasma calcitonin levels (Heath and Sizemore, 1977) and serum alkaline phosphatase (Brewer and Stucky, 1975) than women, while there is a higher count of blood platelets and thrombopoietic activity in plasma of women than men (Kemona et al., 1977).

In the rat, it has long been known that the response to many drugs and xenobiotics is sex dependent. Female animals need only half as much amobarbital as males to be anesthetized, and the sleeping time for a similar dose of the drug is considerably longer in females than in males (Castro and Gillette, 1967; El Defrawy El Masry et al., 1974). This sex difference in drug effect has been attributed to the ability of male rats to metabolize barbiturates faster than female rats. Such sex differences in hepatic metabolism have also been observed for many other xenobiotics, including ethylmorphine, aniline, p-nitroanisole (Quinn et al., 1958; Nicholas and Barron, 1932; Holck et al., 1937) and polychlorinated hydrocarbons (Lamartiniere et al., 1979a).

The sexual differentiation of the levels of several hepatic enzymes appears to be organized during the perinatal period. These enzymes, while maintaining equivalent activities in the prepubertal male and female, undergo differentiation of their levels of

activities at puberty, producing a higher male or female enzyme level in adulthood. DeMoor and Denef (1968) have shown that the sexual differentiation of steroid metabolizing enzymes is dependent on neonatal testosterone secretion, and Gustafsson et al. (1980) have demonstrated that testosterone administered to neonatally castrated animals can program for a male-type steroid metabolism, and we have shown similar results for hepatic monoamine oxidase by using testosterone and diethylstibestrol (Illsley and Lamartiniere, 1980a).

Experimental evidence suggests that gonadotropin secretion, biochemical metabolism, and sexual behavior are controlled via the neuroendocrine system. The fetal and neonatal brain is a developing organ that contains hormone sensitive neurons and the biochemical components necessary for hormone action. The fetal testes begin to synthesize testosterone shortly after the onset of histologic differentiation of the tissue and at the same time that the Leydig cells of the testes appear (Wilson and Siiteri, 1973). Testosterone is the major androgen in plasma and enters target tissues by a passive diffusion process. Inside the brain, target cell testosterone is converted to estrogen via the aromatizing enzyme system (Reddy et al., 1974; Naftolin et al., 1975; McEwen et al., 1977). Neural estrogen receptors in the rat are present in very low amounts just before birth and increase dramatically in the intermediate postnatal period (McEwen et al., 1977; McEwen, 1977; Bardin and Catterall, 1981). Steroid hormone action is subsequently initiated when the steroid hormone binds a cytoplasmic receptor. This hormone-receptor complex is translocated to the cell nucleus (O'Malley and Means, 1974) where it is bound to the chromatin and transcription, translation, and protein synthesis take place to "fire" the nerve endings in the brain to cause sexual differentiation of the brain (Gorski et al., 1978).

In the rat, the target cells are protected against the action of circulating estrogens by alpha fetoprotein which circulates at high concentration during the latter part of gestation and then gradually disappears during the first few weeks of neonatal life (Raynaud et al., 1971; McEwen et al., 1975). This estrogen binding protein does not bind testosterone, leaving testosterone free to enter the target cell where it can be converted to estrogen and interact with cellular estrogen receptors (MacLusky and Naftolin, 1981; McEwen, 1976; McEwen et al., 1975). In the female rat the presence of the ovaries may also prevent the defeminizing effects of neonatal androgen treatment, and perhaps even progesterone serves to protect the developing female fetus from circulating androgens (Shapiro et al., 1976). The specificity of the testosterone derived estrogen is directly involved in the imprinting for

sexual development; this is supported by the fact that the synthetic estrogen, diethylstilbestrol, has a low affinity for alpha fetoprotein and is more potent than estrogen in inducing sexual differentiation of the female rat brain (Slaughter et al., 1977). In addition, dihydrotestosterone (nonaromatizable androgen) is less effective than testosterone or estradiol at inducing defeminization of the neonatal rat brain (MacLusky and Naftolin, 1981).

BEHAVIOR

SEXUAL BEHAVIOR

In our laboratory we have investigated the role of hormones and hormonally-active xenobiotics on sexual behavior. Working with a hormone-controlled behavior pattern allows for a relatively precise study of the neural mechanisms of sex-behavior and reproduction. The natural source of hormone can be removed (gonadectomy) and then systematically replaced by injections or implants. Mating behavior is of obvious biologic importance, and lordosis behavior by the adult female is under strong hormonal control by estrogens and progesterone. The ability to demonstrate lordosis, however, is organized during the critical period of brain development (Dobbing and Sands, 1979; MacLusky and Naftolin, 1981; Ehrhardt and Meyer-Bahlburg, 1981) and has been shown to be dependent on neonatal androgen being converted to estrogen and subsequent programming for a male type of sex behavior (McEwen, 1976). Simply described, lordosis behavior is a series of fixed motor patterns consisting of back flexion, neck extension, lateral tail deviation, and elevation of the perineum upon being mounted by a stud-male. Lordosis was chosen as the test for monitoring alterations of organizational effects on sexual behavior because it is reliable, convenient, informative, and cost-effective (Pfaff, 1979). Our recent work confirms and extends the work of others on the effect of sex steroids (Gorski, 1974) and estrogenically-active xenobiotics on female sexual behavior. Adult test animals (Sprague-Dawley male or female rats) were given s.c. injections of 8 μ g estradiol benzoate/kg BW for 3 days, followed by 500 μ g progesterone/rat on the fourth day to facilitate lordosis. The animals were tested 4 to 6 hours after the progesterone injection. A proestrous intact female or an ovariectomized female demonstrates a high level of lordosis. An intact male (sham operated) or an adult-castrated male demonstrates a low level of lordosis (Figure 1). A male rat castrated within 24 hours following parturition will act like a female i.e. a high lordosis quotient (Figure 1, second bar). However, a neonatally castrated male treated on day 2 with 1.45 μ moles (500 μ g) testosterone propionate is characterized by a significantly reduced lordosis quotient (fourth bar). Treatment with 0.483 μ moles testosterone propionate had no effect on lordosis quotient. Neonatally castrated males treated on day 2 with 0.483 or 1.45 μ moles estradiol benzoate or diethylstilbestrol were characterized by having significantly reduced lordosis quotients.

Not only is a lower dose of estradiol benzoate more effective than testosterone benzoate in lowering lordosis quotient but the nonsteroidal estrogen, diethylstilbestrol, was the most effective in suppressing female behavior.

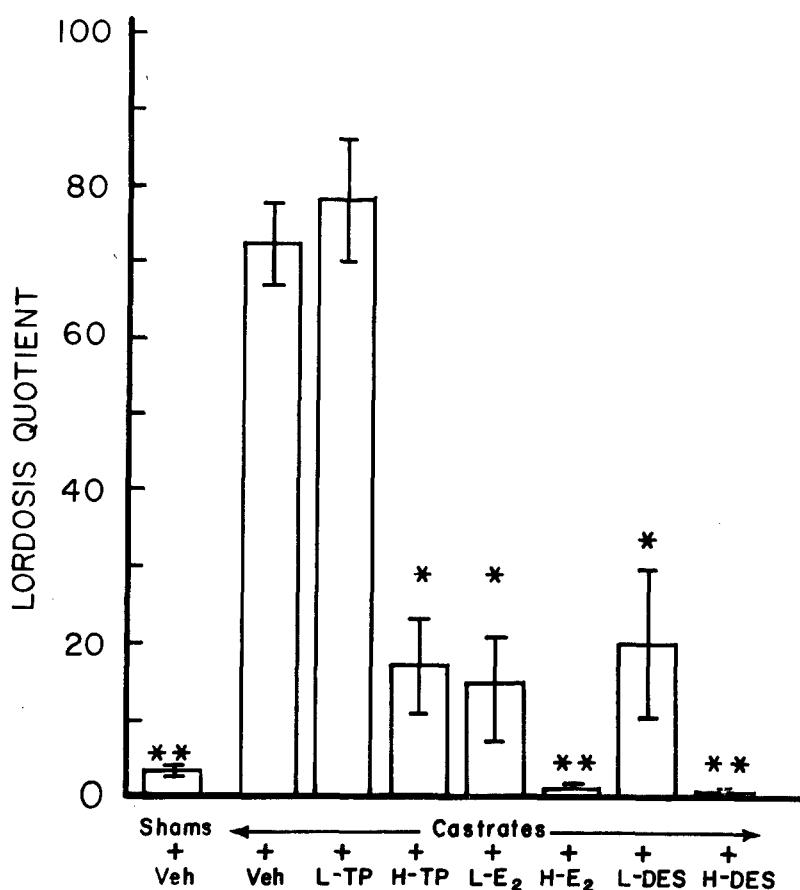


Figure 1. The neonatal effects of hormones on lordosis behavior in adult rats. Rats were castrated or sham operated on day 1 following parturition and given s.c. injections of 20 μ l propylene glycol (Veh) or testosterone propionate (TP), estradiol-17 β (E₂), or diethylstilbestrol (DES) at concentrations of 0.483 μ moles (L) or 1.45 μ moles (H) on day 2 postpartum. Values represent means \pm S.E.M. (n = 9). *P < 0.01; **P < 0.001 when compared to castrates plus vehicle (Mann-Whitney-U test).

ESTROGENICALLY-ACTIVE XENOBIOTICS

Numerous occupational and environmental chemicals have been demonstrated to possess hormone activity. Chemical exposure may be secondary to the useful application and remains of therapeutic substances or growth promoting agents from treated animals, substances contained in modern packing materials, or the residues

from pesticides or herbicides. Estrogenic activity has been demonstrated in a variety of drugs and environmental chemicals. Diethylstilbestrol (Dodds, 1968), polychlorinated biphenyls (Bitman and Cecil, 1970), DDT, (Bitman and Cecil, 1970), methoxychlor (Bitman and Cecil, 1970), zearalenone (Mirocha and Christensen, 1974) and tetrahydrocannabinol (Solomon et al., 1976) elicit a positive uterotrophic response and/or bind competitively to estrogen receptors. Should the developing organism be exposed to hormonally-active xenobiotics during the critical period of brain development, it is conceivable that normal maturational processes might be altered. These hormonally-active xenobiotics might exert this effect by causing estrogenic or antiestrogenic actions.

Two of these environmental chemicals, *o,p'*-DDT and methoxychlor, have been extensively investigated as to their toxic effect and potential hazards as weak estrogenic xenobiotics. The biologically persistent commercial insecticide mixture of DDT contains about 15-20% *o,p'*-DDT, an isomer that has been demonstrated to possess weak estrogenic properties. Methoxychlor, the bis-*p*-methoxy derivate of DDT, is presently being used as a substitute for DDT because it has an appreciably shorter estimated biologic half-life (24 hours versus 10-20 years). The difference in clearance rates in animal systems is reflected by the rapidly metabolized *p,p'*-methoxy substitutes on the phenyl rings of methoxychlor rather than the chlorines of *o,p'*-DDT. A number of laboratories have demonstrated that exogenously administered *o,p'*-DDT or methoxychlor to immature female rodents or to adult ovariectomized rodents will result in a positive uterotrophic response (Bitman and Cecil, 1970; Welch et al., 1969; Clement and Okey, 1972). While it does not appear that methoxychlor, itself, is estrogenic, it appears that the di-demethylated metabolite is 50-fold more potent than the parent compound (Kupfer and Bulger, 1980; Bulger et al., 1978; Nelson et al., 1978). The addition of *o,p'*-DDT to rat uterine cytosol inhibits estrogen binding (Kupfer and Bulger, 1976). It has also been shown that *o,p'*-DDT interferes with the binding of estradiol to the 8S uterine cytosolic receptor and that the inhibition by *o,p'*-DDT appears to be competitive without alteration of the number of total estrogen binding sites (Nelson, 1974; Kupfer and Bulger, 1976). In translocation experiments, *o,p'*-DDT lowered uterine cytoplasmic receptor content and increased nuclear receptors. The duration of this effect was substantially longer with *o,p'*-DDT than with estradiol (Kupfer and Bulger, 1976). The authors suggest that *o,p'*-DDT may be an anti-estrogen as well as an estrogen. In addition to the phenolic metabolite of methoxychlor stimulating a uterine response, it competitively inhibits estradiol binding to the cytosolic receptor. The ligand-receptor complex translocates to the nucleus and stimulates uterine ornithine decarboxylase synthesis (Bulger et al.,

1978). Female rats injected neonatally with o,p'-DDT demonstrate advanced puberty, persistent vaginal estrus, ovaries that develop follicular cysts, and a reduced number of corpora lutea (Heinrichs et al., 1971). Male rats treated neonatally with o,p'-DDT or methoxychlor had normal reproductive organ weights and motile sperm as adults (Gellert et al., 1974). Female offspring from pregnant rats exposed to 1000 ppm methoxychlor had early vaginal openings and reduced reproductive capacity when they attained maturity and were mated (Harris et al., 1974). Males also had impaired reproductive behavior.

Lee and Visek (Lee, 1978; Lee and Visek, 1975) administered 3 mg o,p'-DDT to male rats shortly after birth, castrated them at 3 months and implanted ovarian tissue of immature female rats in the anterior eye chamber. Rhythmic neural gonadotrophic secretion was obtained as evidenced by the capacity to form corpora hemorrhagica and corpora lutea. Their in vitro studies showed that o,p'-DDT inhibits testosterone binding to hypothalamic androgen receptors. The authors suggest that o,p'-DDT inhibits the binding of testosterone and estrogen to their specific receptors in the hypothalamus during the critical period of sexual differentiation to interfere with the sexual differentiation of the brain.

These reports prompted us to investigate the potential of o,p'-DDT and methoxychlor to elicit organizational effects on the developing rodent brain, effects that would be expressed in adulthood via sexual behavior. We designed our experiments to elucidate the mechanism of action of these xenobiotics in the presence and absence of hormones, in neonatally castrated rats, and in intact rats. We wished to determine if these estrogenically-active xenobiotics would exert a developmental effect by acting as estrogens (defeminization: low lordosis quotient) or antiestrogens (binding the estrogen receptors and prohibiting defeminization). As discussed in the previous section, a neonatally sham-operated male demonstrates a low lordosis quotient while a neonatally castrated male demonstrates a high lordosis quotient (Figure 2). Neonatally castrated and o,p'-DDT treated rats (1 mg or 3 mg/rat on days 2, 4, and 6 postpartum) tested for female sexual behavior had high lordosis quotients. Similar treatment, however, with methoxychlor results in a reduction in lordosis quotient i.e. defeminization. Both doses were effective in reducing lordosis quotient, the higher dose (3 mg/rat) causing a statistically significant effect.

In animals castrated and treated neonatally with both 1.45 μ moles testosterone propionate and 3 mg/rat o,p'-DDT, lordosis quotients of 15 ± 9.6 were obtained, values that are similar to ones obtained for neonatal castrates plus testosterone propionate only. Neonatal castrates given testosterone propionate and 3 mg/rat methoxychlor had lordosis quotients of 2.1 ± 2.1 , i.e. it

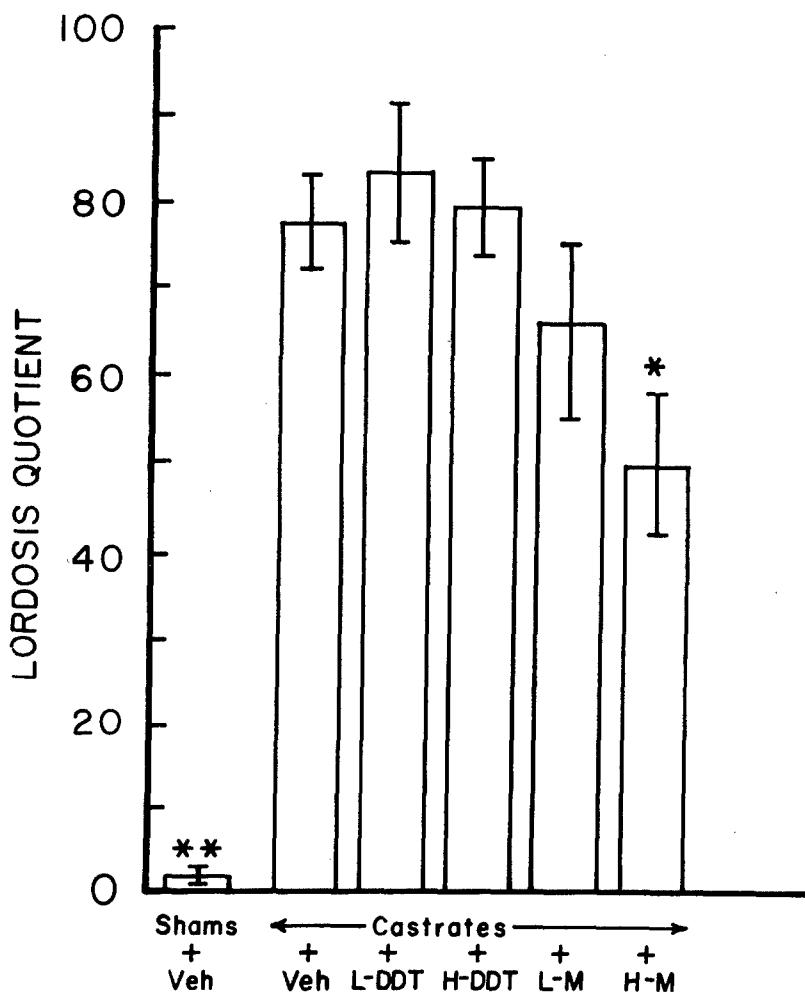


Figure 2. The neonatal effects of estrogenically-active xenobiotics on lordosis behavior in adult rats. Rats were castrated or sham operated on day 1 following parturition and given s.c. injections of 20 μ l sesame oil (Veh), or o,p'-DDT (DDT), or methoxychlor (M) at doses of 1 mg (L) or 3 mg (H) per animal on days 2, 4, and 6 postpartum. Values represent means \pm S.E.M. (n = 9). *P < 0.05; **P < 0.001 when compared to castrates + vehicle (Mann-Whitney-U test).

appears that the exogenous administration of both testosterone and methoxychlor resulted in an additive effect toward defeminization of female sexual behavior. In any case, the two environmental estrogens (o,p'-DDT and methoxychlor) did not exert an antiestrogenic effect toward defeminization of sexual behavior. It therefore appears that neonatal exposure to methoxychlor, as to testosterone, results in organizational effects that are expressed in adulthood, an effect that is apparently permanent and expressed in the absence

of the original effector. The finding that an environmental chemical that is used routinely in the home garden but possessing weak estrogenic capability could cause such an effect on sexual behavior in animals is profound and merits further investigation of other hormonally-active xenobiotics and into the effects that these chemicals have on the intact animal.

Towards the latter parameter we have studied the effects of neonatal exposure of intact rats to testosterone, diethylstilbestrol, o,p'-DDT, and methoxychlor on sexual behavior and hepatic metabolism. For the sexual behavior studies, newborn male rats were treated on days 2, 4, and 6 with 3 mg o,p'-DDT or methoxychlor or 1.45 μ moles testosterone propionate on day 4 or with 1.45 μ moles diethylstilbestrol on day 2. The latter two treatments were included since previous reports demonstrated that neonatal exposure to testosterone and diethylstilbestrol had an adverse effect on gonadal development. Diethylstilbestrol reduced spermatogenesis and lowered concentrations of circulating testosterone levels (Lamartiniere et al., 1979b; Lamartiniere and Lucier, 1981). These animals were subsequently castrated 10 weeks later and tested for female sexual behavior at 16 weeks of age. All of these animals had lordosis quotients similar to those of sham-operated males (results not shown) i.e. hormones and estrogenically-active xenobiotics administered neonatally to intact male rats did not interfere with the normal defeminizational process of sexual behavior.

EFFECTS OF HORMONES ON MORPHOMETRIC MEASUREMENTS

Neonatal administration of diethylstilbestrol and estradiol-17 β but not testosterone propionate to intact male rats also significantly altered the adult body weights, grip strength scores and emergence latencies (a measure of approach to novelty) of these animals and were similar to females given vehicle only (Tilson and Lamartiniere, 1979). The adult body weights, grip scores, and emergence latencies of male pups castrated at birth were similar to those of female rats and these effects were not altered by neonatal administration of diethylstilbestrol or estradiol-17 β . Neonatal administration of testosterone propionate appeared to block the effect of castration on body weights and grip strength scores but did not alter emergence latencies. Thus, intact males given diethylstilbestrol or estradiol-17 β and castrates given vehicle, diethylstilbestrol or estradiol-17 β did not differ significantly from intact females. These data suggest that the presence of high levels of estradiol-17 β and diethylstilbestrol during the period of sexual differentiation can feminize or demasculinize certain male characteristics (Tilson and Lamartiniere, 1979).

HEPATIC METABOLISM

The major focus of our research has been on the effects that perinatal exposure to hormones and xenobiotics have on the ontogeny and endocrine regulation of hepatic metabolism. In particular, we have studied the role of the hypothalamic pituitary gonadal axis on the initiation and maintenance of sexual differentiation of hepatic enzymology and the toxicologic mechanisms exerted by xenobiotics. The postnatal developmental pattern of most of these enzyme systems is characterized by low catalytic activities during the neonatal period, a gradual increase in activity during the prepubertal period, followed by postpubertal sexual differentiation resulting in higher activity levels being attained in one sex or the other.

ONTOGENY AND ENDOCRINE REGULATION OF HEPATIC ENZYMES

MONOAMINE OXIDASE

Hepatic monoamine oxidase is a mitochondrial biogenic amine metabolizing enzyme system that is sexually differentiated such that adult female rats have higher activities than adult male rats. Adult castration of males results in higher activities of monoamine oxidase, while exogenously administered testosterone reverses the effect of castration (Illsley et al., 1980). Hypophysectomy of male and female rats results in elevated levels of monoamine oxidase and an ectopic pituitary transplanted under the kidney capsule of a hypophysectomized female rat reverses the effect of hypophysectomy. An ectopic pituitary with or without exogenous testosterone in a hypophysectomized male has no effect on monoamine oxidase activity. Our results suggest that hepatic monoamine oxidase activity is partially regulated by a pituitary factor or factors, as yet unidentified, which suppress enzyme activity. In the adult female, release of this pituitary factor appears to be independent of hypothalamic control. Release of this factor in the male seems to be dependent on the hypothalamus and stimulated by testosterone (Illsley et al., 1980). We have also investigated the possibility of neonatal imprinting of hepatic monoamine oxidase (Illsley and Lamartiniere, 1980). Monoamine oxidase activity in the adult male, normally lower than that in the adult female, was elevated by neonatal castration to a level similar to that in the adult intact female. The increased activity due to neonatal castration was not an artifact of adult androgen deprivation, since administration of testosterone propionate or dihydrotestosterone to the adult (castrated neonatally) for 7 days before sacrifice failed to reverse this increase. The administration of testosterone propionate or diethylstilbestrol to neonatal castrates on day 2 prevented the rise in activity observed in the adult male after neonatal castration.

These results suggest that the sex-related differences in adult male and female monoamine oxidase activities and the androgen responsiveness of male adult activity are imprinted during the neonatal period. The natural imprinting agent appears to be an androgen, like testosterone, which might act intracellularly as an estrogen (Illsley and Lamartiniere, 1980).

GLUTATHIONE S-TRANSFERASES

The glutathione S-transferases are a group of cytosolic proteins that catalyze many reactions in which glutathione participates as a nucleophile. These enzymes bind a large number of hydrophobic compounds and act as a storage facility prior to metabolism or excretion of the ligand (Jakoby and Keen, 1977) and undergo covalent-bond formation with reactive electrophilic carbons (Keen and Jakoby, 1978). The glutathione S-transferases have been separated on the basis of differences in physical properties, and while individual proteins have been found to catalyze reactions with more than one class of substrate, each protein displays preferred specificity (Habig et al., 1974). In our studies, we measure glutathione S-transferase activities as a function of reduced glutathione conjugation towards the substrates 1-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene (Lamartiniere et al., 1979; Habig et al., 1974; Nemerooff et al., 1977).

Glutathione conjugation toward these two substrates indicates that glutathione S-transferase activities are low in the livers of prepubertal male and female rats. While activities continue to rise in animals of both sexes, conjugation towards 1-chloro-2,4-dinitrobenzene is slightly higher in adult males than in adult females and 2-3 fold higher towards 1,2-dichloro-4-nitrobenzene in males than in females. Sex steroids in the adult animal do not seem to play a role in the postpubertal sex differences of the glutathione S-transferases. Gonadectomy of adult male or female rats with or without appropriate hormone replacement produces no significant change in enzyme activities (Lamartiniere, 1981). On the other hand, the pituitary is responsible for negative modulation of the glutathione S-transferase activities (feminization). Hypophysectomy results in increased glutathione S-transferase activities. To gain insight into the nature of this hypophyseal regulation, we implanted a pituitary from an adult age-matched donor or 4 pituitaries from 21-day old males (equivalent weight) to hypophysectomized animals. The animals were sacrificed eight days later. These ectopic pituitary treatments were capable of reversing the effect of hypophysectomy on glutathione S-transferase activities (Lamartiniere, 1981). Similar results were obtained in female rats. The increase in glutathione S-transferase

activities in hypophysectomized animals was followed by a decrease in serum prolactin concentrations and the reverse was found in those hypophysectomized animals receiving ectopic pituitaries; i.e., a decrease in enzyme activity and increase in prolactin. This led us to investigate the possibility of prolactin mediating the regulation of the glutathione S-transferases. Rat prolactin (N.I.A.M.D.D.-Rat Prolactin-B1, 7 units/mg) was given by subcutaneous injections in saline to hypophysectomized rats at concentrations of 780 μ g/kg BW twice daily for 8 days. This prolactin treatment was not capable of altering glutathione S-transferase activities but was capable of stimulating accessory sex organ weights (Lamartiniere, 1981). A similar experiment with growth hormone as a possible hypophyseal effector was carried out. Bovine growth hormone (N.I.H. GH-B-18, 0.83 units/mg) administered under the same schedule as prolactin at a concentration of 5 mg/kg BW was able to reverse the effect of hypophysectomy on glutathione S-transferase activities (Lamartiniere, 1981). This demonstrates an inverse relationship between glutathione S-transferase activities and growth hormone levels.

In an effort to gain further insight into the mechanisms of the hypothalamic-hypophyseal regulation of glutathione S-transferases, arcuate nucleus lesions of the hypothalamus were induced by injecting neonatal rats with pharmacologic doses of monosodium-L-glutamate according to the methods of Nemeroff et al. (1977). Light microscopic examination of Nissl-stained sections of the medial basal hypothalamus of neonatal rats shortly after monosodium-L-glutamate injections revealed considerable damage in the region of the arcuate nucleus (Nemeroff et al., 1977). Glutathione conjugation towards 1,2-dichloro-4-nitrobenzene in adult males treated neonatally with monosodium-L-glutamate was reduced to activities comparable to control females, whereas activity in females was only slightly reduced. Glutathione conjugation towards 1-chloro-2,4-nitrobenzene was not significantly altered in males or females.

Certain experiments (i.e. hypophysectomy with or without an ectopic pituitary or exogenously administered pituitary hormones, neonatal castrations, or chemical lesioning of the arcuate nucleus of the hypothalamus) suggest that prolactin, growth hormone, or a novel pituitary hormone may play a role in the hormonal regulation of glutathione S-transferases by concerted action with other peripheral organs such as the thyroid or adrenals, or act directly on the liver to mediate specific protein synthesis of hepatic peptides. One such peptide is somatomedin (VanWyk and Underwood, 1975). It is synthesized in the liver. Prolactin and growth hormone are also independently and collectively positive modulators of hepatic somatomedin. We therefore had prolactin, growth hormone, and somatomedin concentrations determined in control and monosodium L-glutamate-treated male rats. Prolactin,

growth hormone and somatomedin concentrations as well as glutathione conjugation towards 1,2-dichloro-4-nitrobenzene were significantly reduced in adult male rats treated neonatally with monosodium L-glutamate, as compared to the control animals.

<u>Treatment</u>	<u>Glutathione S-transferase (DCNB**)</u>	<u>Prolactin (ng/ml)</u>	<u>Growth Hormone (ng/ml)</u>	<u>Somatomedin* (unit/ml)</u>
Saline	118 ± 3	21.5 ± 4.0	56.4 ± 11.8	1.98 ± 0.18
Monosodium L-glutamate	58 ± 4	7.6 ± 0.8	4.0 ± 0.2	0.38 ± 0.07

* Assayed in the laboratory of Dr. J. VanWyk (1977).

** (DCNB) 1,2-dichloro-4-nitrobenzene.

Summarizing our experimental data on the endocrine regulation of the glutathione S-transferases, we show that exogenously administered prolactin does not reverse the effect of hypophysectomy on glutathione S-transferase activities. Coupling this with the fact that low prolactin concentrations were found with low glutathione S-transferase activities in adult rats that were neonatally castrated or treated neonatally with monosodium L-glutamate, and the inverse relationship between prolactin concentrations and glutathione S-transferase activities in hypophysectomized rats with or without ectopic pituitaries, we rule out prolactin as being responsible for the feminization of glutathione S-transferases. In contrast, exogenously administered growth hormone is capable of reversing the effect of hypophysectomy on the glutathione S-transferases (decreases activity); however, decreased glutathione S-transferase activities in adult male rats treated neonatally with monosodium L-glutamate are paralleled by significantly decreased growth hormone concentrations, therefore suggesting that growth hormone is also not the feminizing factor. Since somatomedin concentrations were reduced when glutathione S-transferase activities were decreased, and because somatomedin regulation is dependent on prolactin and growth hormone levels (VanWyk and Underwood, 1975), it appears doubtful that this peptide is the feminizing factor. Certainly, further experiments are necessary to elucidate the nature of the specific factor(s) involved in the endocrine regulation of the glutathione S-transferases.

UDP-GLUCURONYLTRANSFERASE

The UDP-glucuronyltransferases are located in the membrane of the endoplasmic reticulum and catabolize the conjugation of glucuronic acid to compounds containing hydroxyl, carboxyl, amino, imino, and sulfhydryl groups, consequently rendering these

products more hydrophilic and more readily excretable. Considerable evidence has accumulated in the literature to demonstrate multiple forms of UDP-glucuronyltransferases. One class has been characterized as belonging to the "late-fetal group" (Wishart, 1978) and conjugates mostly non-steroidal compounds (Lucier and McDaniel, 1977), while the other class is of the "neonatal group" (Wishart, 1978) and conjugates mostly steroids (Lucier and McDaniel, 1977). This report deals with the former group due to the ease and sensitivity of measuring enzyme activity with p-nitrophenol as substrate. While prepubertal UDP-glucuronyltransferase activity is similar in male and female rats, postpubertal sex differentiation results in male activities that are nearly twice that of females (Lamartiniere et al., 1979). Postpubertal castration of male rats results in reduced enzyme activity whereas ovariectomy of females causes no significant alteration in activity levels. Testosterone propionate (2 mg/kg BW for a week) administered to castrate males is capable of restoring UDP-glucuronyltransferase activity to normal levels. Hypophysectomy of male rats results in a decrease in enzyme activity, whereas hypophysectomy has no effect on female UDP-glucuronyltransferase. Hypophysectomy therefore abolishes postpubertal sex-difference of this enzyme activity. A pituitary transplant under the kidney capsule with or without exogenous testosterone treatment is not capable of reversing the effect of hypophysectomy on UDP-glucuronyltransferase activity (Lamartiniere et al., 1979). These experiments demonstrate that testosterone and the pituitary are positive modulators of UDP-glucuronyltransferase activity, but an *in situ* pituitary is obligatory for androgen action. Furthermore, it would appear from the pituitary transplant experiments that the positive modulation by the pituitary is under the control of a hypothalamic "releasing factor". Exogenously administered prolactin or growth hormone to hypophysectomized rats was not capable of reversing the effect of hypophysectomy on enzyme activity.

PERINATAL EXPOSURES TO HORMONES

We have investigated the effect that neonatal hormone exposure on intact rats would have on hepatic sex-differentiated enzymes in the adult animal. Newborns were treated on days 2, 4, and 6 postpartum with 1.45 μ moles testosterone propionate, diethylstilbestrol, or 20 μ l propylene glycol (vehicle). Adult males that had received the hormones neonatally had significantly lower UDP-glucuronyltransferase activities than the controls (Lamartiniere et al., 1979). Neonatal testosterone propionate or diethylstilbestrol treatment had no effect on UDP-glucuronyltransferase activity in prepubertal males or females or adult females. Glutathione S-transferase activities were unaltered in these treatment groups. Neonatal treatment with testosterone

propionate and diethylstilbestrol resulted in reduced reproductive organ weights in adult male and female rats, but only neonatal diethylstilbestrol had an adverse effect on spermatogenesis and on circulating testosterone levels. It would appear that the feminization of UDP-glucuronyltransferase activities in those neonatally treated males must reflect more than a reduction in androgen levels. Apparently, there are other alterations from this treatment that are responsible for reduced enzyme activity in diethylstilbestrol and testosterone propionate treated animals (Lamartiniere et al., 1979).

Neonatal treatment with testosterone propionate had no effect on body weights of 21-day-old and 63-day-old male rats, but at 97 days male body weights were significantly greater than propylene glycol control values (Lamartiniere et al., 1979). Diethylstilbestrol treatment resulted in significantly lower body weights throughout the study period. An investigation on the effects of neonatal exposure to these hormones on the male reproductive tract revealed a pronounced decrease in testicular weights at 21, 63, and 97 days of age. Diethylstilbestrol had a greater effect on testicular size than testosterone propionate. Histologic observations of testes from 63-day-old animals demonstrated decreased spermatogenic activity in the diethylstilbestrol-treated males, whereas testosterone propionate treatment had no observable effect on spermatogenesis. Serum testosterone concentrations in these adult male rats that had been given propylene glycol, testosterone propionate or diethylstilbestrol neonatally were 2.5 ± 0.3 , 3.2 ± 0.3 , and 0.5 ± 0.1 (mean \pm S.E.M.) ng/ml, respectively. Weights of accessory sex organs in adult male rats were also changed by neonatal treatment with testosterone propionate or diethylstilbestrol. Seminal vesicle weights of adult testosterone propionate- and diethylstilbestrol-treated males were only 38% and 11%, respectively of those of propylene glycol-treated males. Ventral prostate weights of testosterone propionate- and diethylstilbestrol-treated males were 60% and 32% of the values of the controls. Neonatal diethylstilbestrol or testosterone propionate treatment delayed testicular descent, which occurred at 21-28 days in controls, in contrast with 35-42 days in testosterone propionate-treated males and 42-63 days in diethylstilbestrol-treated males (Lamartiniere et al., 1979).

Neonatal treatment with pharmacologic doses of diethylstilbestrol, estradiol-17 β or testosterone propionate results in decreased uterine weights in adult females (Lamartiniere, 1979). Neonatal diethylstilbestrol and estradiol-17 β also result in precocious puberty in females, while neonatal testosterone retards vaginal opening through at least 105 days of age. Circulating sera estrogen levels were lower in adult diethylstilbestrol and estradiol-17 β -treated females than in testosterone-treated and control females.

PERINATAL EXPOSURE TO XENOBIOTICS

Our laboratory has investigated the perinatal effects of a number of xenobiotics on postnatal development. We have found that low doses of diethylstilbestrol administered during gestation causes masculinization (decrease in activity) of hepatic histidase activities in adult female rats (Lamartiniere and Lucier, 1978). Gestational exposure to 5,5-diphenylhydantoin causes hepatic monoamine oxidase activities in the adult male offspring to be elevated to levels similar to that of adult females (Illsley and Lamartiniere, 1979). The serum levels of testosterone in the male offspring of prenatally treated females on days 5 and 63 postpartum were the same as those of their respective controls, demonstrating that the changes caused by diphenylhydantoin are not due to diminished levels of testosterone. We suggest that diphenylhydantoin may interfere with the perinatal programming of hepatic monoamine oxidase (Illsley and Lamartiniere, 1979).

Using two pure polychlorinated biphenyl congeners, we have also investigated the effects of perinatal exposure of the 3,4-3',4'tetrachlorobiphenyl (4-CB) and the 2,4,5-2'4',5 hexachlorobiphenyl (6-CB) on two xenobiotic conjugative enzymes (glutathione S-transferase and UDP-glucuronyltransferase) (Lamartiniere et al., 1979; Lamartiniere and Lucier, 1981). Date-bred rats were treated orally on alternate days from the 8th through the 18th day of gestation, with either 3 mg/kg 4-CB or 30 mg/kg 6-CB. These doses did not cause fetotoxicity or decreased survival rate. Neonatal, prepubertal and adult male and female rats exposed perinatally to 6-CB had higher glutathione S-transferase activities than the controls (Lamartiniere et al., 1979). In contrast, perinatal 6-CB exposure does not alter prepubertal and adult female UDP-glucuronyltransferase, but it does decrease adult male enzyme activities (Lamartiniere and Lucier, 1981). The metabolism and disposition of pure PCB congeners differ according to the degree and position of chlorination. The 6-CB does not have the configuration required for rapid hydroxylation and has a long biologic half-life. This may account for the ability to stimulate glutathione S-transferase activities in all ages investigated, but it may be through another mechanism that perinatal 6-CB causes feminization of adult male UDP-glucuronyltransferase. One possibility is that neonatal exposure to PCBs alters activity of steroid-metabolizing enzymes during the critical period of early development when organizational effects are taking place. This could cause alterations in the hormonal milieu during the period of early development resulting in altered sex differentiation of hepatic enzymes. Reports in the literature indicate that PCBs do alter activities of steroid-metabolizing enzymes in adult rats (Lamartiniere and Lucier, 1981; Dieringer et al., 1979).

As pointed out in the first section, hormonally-active xenobiotics can alter sexual behavior in rodents by exerting an organizational effect during the critical period of brain development resulting in permanent and irreversible alterations that are expressed in adulthood. But can perinatal exposure to these estrogenically-active xenobiotics cause a similar effect on adult hepatic metabolism to result in altered ontogeny of hepatic enzymology? To investigate this we treated intact male and female rats during the neonatal period with *o,p'*-DDT or methoxychlor and measured monoamine oxidase, glutathione S-transferase, and UDP-glucuronyltransferase activities and P-450 content in the resulting animals before and after enzyme sexual differentiation (Lamartiniere et al., 1981). Neonatal administration of *o,p'*-DDT or methoxychlor to intact rats resulted in elevated levels of hepatic monoamine oxidase activities in adult rats, but not in prepubertal animals. This treatment did not alter glutathione S-transferase and UDP-glucuronyltransferase activities or cytochrome P-450 content in adult rats, but glutathione S-transferase activities and cytochrome P-450 content were higher in prepubertal animals treated neonatally with *o,p'*-DDT. No significant changes in testes, seminal vesicle, ventral prostate, or body weights were observed in these xenobiotic-treated animals versus the controls. We attribute the changes in prepubertal glutathione S-transferase activities and cytochrome P-450 content to induction by the xenobiotic *o,p'*-DDT. It appears, however, that the ability of *o,p'*-DDT and methoxychlor to alter the sex-differentiation of hepatic monoamine oxidase may be a consequence of changes in the hormonal milieu during a critical period of neonatal development. This increase in adult monoamine oxidase activities was demonstrated only following exposure to these xenobiotics during the first week following parturition and not following treatment during the third week or in adulthood (Lamartiniere et al., 1981). Since *o,p'*-DDT and methoxychlor have estrogenic activity (Bitman and Cecil, 1970; Welch et al., 1969), we surmise that these hormonally-active xenobiotics may reach the brain, bind hypothalamic estrogen receptors (Lee, 1978; Lee and Visek, 1975), and consequently interfere with normal maturational processes important to sex-differentiation of the hypothalamus (Illsley and Lamartiniere, 1980; Gorski et al., 1978).

CONCLUDING REMARKS

In the rat, as in man, maturation of the brain during the perinatal period of development is paramount to the final expression of sexual differentiation. Under "optimum conditions", "normal" male or female phenotypes will develop. However, changes in the normal brain endocrine environment may produce changes in endocrine secretion, behavior, reproduction, and hepatic function. Manipulative surgery and/or steroid therapy during critical period of brain development has been used as a probe to demonstrate organizational effects on these sex-differentiated parameters. There is a dynamic balance of timed endocrinologic events involved

in the ontogeny and homeostasis of the maturing organism, and exposure of the brain or target organ to chemicals during this critical period of development can exert permanent irreversible manifestations that can cause alterations in the offspring's postnatal development. These alterations may be expressed overtly as teratogenesis or a disease state or subtly as alterations in reproductive capacity, sexual behavior, endocrine secretion, or the capacity to metabolize xenobiotics. For example, Miller et al. (1961) reported that the liver of male rats is more susceptible to carcinogenic aromatic amine derivatives than the liver of female rats. Weisburger et al. (1966) treated female rats on the day of parturition with estrogen and shortly after weaning with the carcinogen N-hydroxy-N-2-fluorenylacetamide. Necropsies 26 weeks later showed a higher incidence of liver cancer in the estrogen and carcinogen treated animals than in control animals. The authors suggested that endocrine and possibly central nervous-system factors may play a role in formation of liver tumors. It has also been reported that neonatal exposure to pharmacologic doses of phenobarbital produces a permanent and irreversible modification of the adult hepatic monooxygenase system and the rate of formation of DNA adducts to aflatoxin metabolites (Faris and Campbell, 1981). These researchers theorized that phenobarbital altered the hormonal environment during the critical period of early development and thereby affected brain differentiation in a way that permanently altered pituitary regulation of the hepatic monooxygenase system.

It therefore appears that steroid hormones and xenobiotics are capable of exerting permanent irreversible modifications early in life that may result in alterations in endocrine secretion, behavior, reproduction or predispose the individual to cancer or to other biochemical insult.

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MALE VS. FEMALE MEDIATED TERATOGENESIS

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INTRODUCTION

The purpose of this presentation is to correlate different types of reproductive dysfunction with exposure of males and females during different stages of the reproductive process. It is a well-established principle that teratogenic exposure of the pregnant female, and thus of the embryo, during the organogenesis period of development can lead to birth defects in the offspring. In this presentation, I will discuss the outcome of this exposure paradigm, as well as the outcomes associated with exposure during other stages of the reproductive process that have received less experimental attention. Insult to the germ cells of the male during spermatogenesis and to the female during oogenesis can also lead to reproductive dysfunction. The type of adverse outcome, however, is quite different from that occurring with embryonic exposure during the organogenesis period. An overview of this area will be given with the intent of focusing on those types of reproductive dysfunction that are most relevant to environmental exposure of men and women.

SUSCEPTIBILITY OF THE NON-PREGNANT AND PREGNANT FEMALE TO REPRODUCTIVE IMPAIRMENT

MATURATION OF THE FEMALE REPRODUCTIVE SYSTEM

Studies of reproductive impairment in non-pregnant females have focused on effects on the process of oogenesis. This process is initiated during fetal life, after the ovary is formed. Figure 1 contains a diagram of the stages of oogenesis, and traces the process from fetal life, through puberty, to fertilization in the reproductively mature adult. During fetal life, oogonia undergo active mitotic proliferation, resulting in the formation of a maximum of 7×10^6 cells. At approximately 3 months of human fetal development, the oogonia enter into prophase I of meiosis in waves, and by 5 months, the entire population has ceased mitotic activity and has begun meiosis as primary oocytes. Extensive physiologic

degeneration of germ cells occurs during the oogonial and primary oocyte stages of development. It has been estimated in humans that 60% of the germ cells present in the 5-month old fetus are lost before birth. Once meiosis is initiated, germ cells lost to physiologic atresia cannot be replaced in the female, unlike the male (see below). Meiotic cells lose the ability to undergo mitosis, and thus to replace lost cells through compensatory hyperplasia. The total number of germ cells potentially available for ovulation in the female offspring is fixed in the fetal period when oogonia mature into primary oocytes, and this number continues to decrease due to physiologic atresia (Hertig and Barton, 1976).

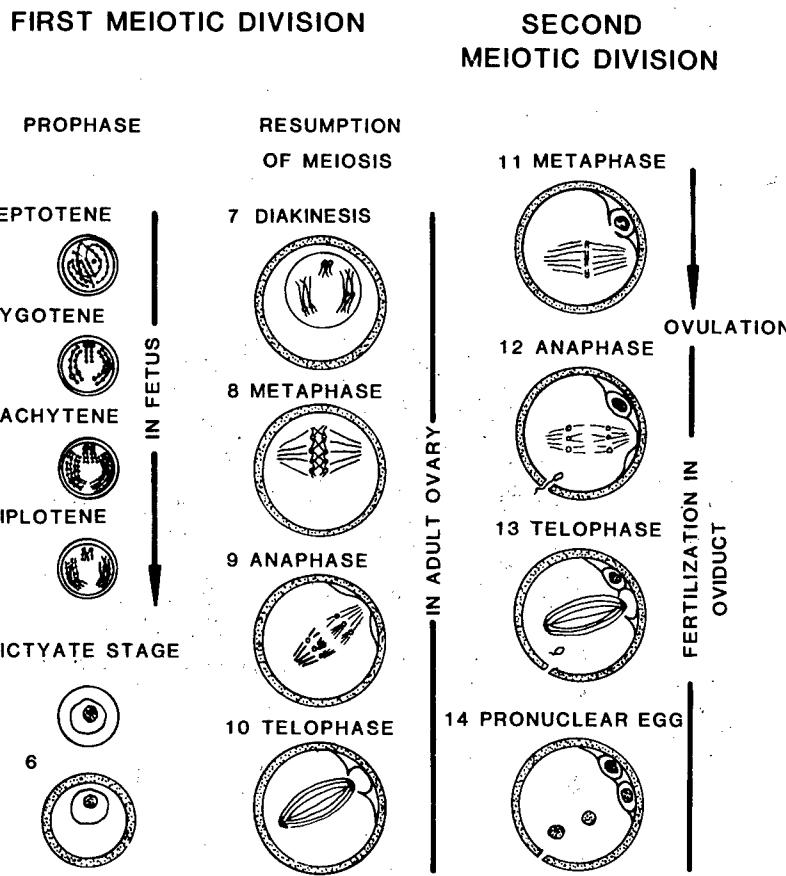


Figure 1. Oocyte maturation. Prophase of the first meiotic division (1-4) occurs during fetal life. At zygotene, homologous chromosomes pair, and at pachytene, they form bivalents. Interchange of genetic material occurs by crossing over. At diplotene, the chromosomes remain united at the points of interchange, the chiasmata. The meiotic process is arrested at the dictyate stage. When meiosis is resumed, the first division is completed (7-11). Ovulation occurs at metaphase of the second division (11), which is completed in the oviduct (12-13) following sperm penetration. Adapted from Tsafriri, 1978.

During the entire span of postnatal life, including the pre-pubertal and reproductive periods, the majority of germ cells in the ovary remain as primary oocytes enclosed with unilamellar follicles (Figure 2). These "primordial follicles" comprise the pool from which a select number of oocytes are recruited for further maturation to pre-ovulatory or Graafian follicles. This maturation process consists of formation of the follicle and growth of the oocyte, and not of progression through meiosis (dictyate stage in Figure 1). At puberty, and within each menstrual cycle thereafter, release of gonadotropins, particularly luteinizing hormone (LH), initiates the resumption of meiosis. The primary oocytes in pre-ovulatory follicles progress through the rest of the first meiotic division, and block in metaphase of the second meiotic division. The secondary oocyte is ovulated in metaphase II, and stays in this state until fertilization occurs. At fertilization, the second meiotic division is completed, the second polar body is extruded, and the female pronucleus is formed, which combines with the male pronucleus to initiate development (Espey, 1978). In the

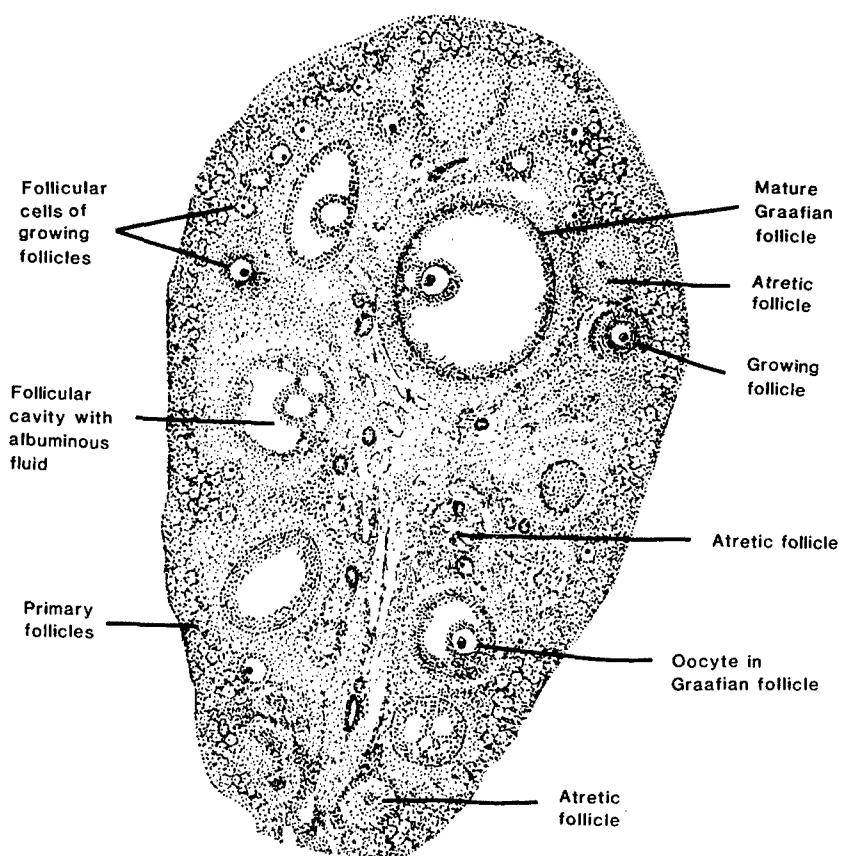


Figure 2. Adult ovary depicting primary (primordial) follicles, growing follicles and mature Graafian follicles.

absence of fertilization, the ovulated secondary oocyte degenerates. When the menstrual cycle is established, ovulation occurs on the average of every 28 days, during which time those follicles recruited up from the primordial follicle pool (pre-ovulatory oocytes) are stimulated to ovulate by elevation in gonadotropin levels. This process continues throughout the reproductive lifespan until the population of primordial follicles is depleted, and/or menopause occurs.

EFFECTS OF CHEMICAL AND PHYSICAL AGENTS ON OOGENESIS

Very few chemical and physical agents have been studied for their effects on the non-pregnant female reproductive system. More information exists for ionizing radiation than for chemical insult, and this information will be used as a model for chemical effects (Baker, 1978). Table 1 contains a summary of the effects of irradiation exposure at various stages of oogenesis. The radio-sensitivity of germ cells varies considerably with age, stage of mitosis or meiosis, and size of the follicle. Primordial germ cells are relatively resistant to radiation administered before or during their migration to the gonad, but become sensitive during sexual differentiation of the gonad. Sensitivity increases to a maximum during the peak wave of mitosis in oogonia, especially during the final interphase before cells enter meiosis. While cells in the migratory and mitotic phases of maturation that are killed by radiation can be replaced by compensatory hyperplasia, once cells have entered meiosis, restoration of dead cells is not possible. Radiation exposure during this time appears to accelerate physiologic atresia, resulting in a reduction in the number of cells that enter meiosis. With the onset of the period of arrested development, sensitivity to radiation-induced cell killing increases. Oocytes in primordial follicles are highly sensitive to cell killing, more so than those in growing follicles. Pre-ovulatory oocytes in multilayered follicles are relatively resistant to cell killing, but are sensitive to mutation induction. In irradiated pre-ovulatory oocytes, the incidence of dominant lethal mutations is highest at metaphase I, slightly less at metaphase II, and low at other stages (Baker, 1976).

A similar pattern of response has been observed with chemical agents, specifically antifolates and alkylating agents (Hansmann and Rohrborn, 1973; Hansmann, 1974). The larger the follicle becomes in preparation for ovulation, the more likely that mutations will be induced rather than cell death. Limited studies have also been conducted on polycyclic hydrocarbons which indicate that these compounds are capable of inducing primordial oocyte destruction in the ovaries of adult mice (Mattison and Thorgeirsson, 1978).

TABLE 1. RADIOSENSITIVITY DURING MAMMALIAN OOGENESIS

(1,700, 3 weeks)	PRIMORDIAL GERM CELLS	
	Proliferation & migration to gonad	
	Cell death, compensatory hyperplasia	
(7 x 10 ⁶ , 6 weeks)	OOGENIA	
	Colonization of gonad	
	Atresia	
(2 x 10 ⁶ , birth)	PRIMARY OOCYTES	
	Atresia, meiosis arrested in Prophase I, dictyate	
	Primordial follicle - high sensitivity, death cell	
(FSH, LH)	SECONDARY OOCYTE	
	Meiosis progresses to metaphase I	
	Preovulatory follicle - highest level mutations, lowest level cell death	
OVULATION	OVUM	
	Meiosis progresses to metaphase II	
	Mutation induction no cell death	
(400-500)		

The total number of germ cells available at each stage of oogenesis is given on the far left and landmarks to the left of the center. Responses to radiation exposure are described on the right of the center. Adapted from Baker, 1978.

A spectrum of reproductive outcomes can occur with exposure of the female germ cells to toxic agents. With transplacental exposure of the fetus, the most likely outcome is reduced fertility in postnatal life. The cell killing of oogonia and early prophase I oocytes by radiation, alkylating agents, and anti-folates can result in an acceleration of physiologic atresia and a decrease in the size of the primordial follicle pool from which pre-ovulatory oocytes are recruited. This can result in subfertility or complete sterility of the offspring. With exposure of the adolescent or reproductively mature adult, it is clear that fertility span is dependent on oocyte number. Animals exposed to near-sterilizing doses of radiation usually have one or two litters resulting from survival of oocytes that were in the process of follicular growth, and thus relatively resistant to radiation. When these oocytes

have been used, the animal becomes sterile due to destruction of the primordial follicle pool. In primates, including humans, therapeutic irradiation of the pelvic region of younger women results in temporary amenorrhea, while in older women, premature onset of menopause can occur (Baker, 1976).

In addition to reduced fertility from oocyte destruction, a variety of genetic effects are also possible that can be passed on to the offspring. These effects could only occur if a mutated ovum participated in fertilization. The Russells (1956, 1972) studied the effects of radiation exposure to adult females on pregnancy outcome by mating female mice at weekly intervals for 7 weeks after acute exposure to a near-sterilizing dose of radiation. Subfertility and mutations in the offspring (dominant lethal and recessive) were characteristic of matings occurring 1 week after exposure, in which ovulated ova and secondary oocytes were sampled. The mutation rate in weeks 2-6 of mating, in which oocytes exposed in immature growing follicles were sampled, was higher than at week 1 (mature follicles). Matings from 7 weeks onward were often subfertile, but mutations were not found in litters of those females that did conceive at this time interval when primordial follicles were sampled. These results are summarized in Table 2. The earlier the stage of oogenesis, the more likely that cell killing and thus that infertility will occur. At later stages, especially those approaching ovulation, the more likely it is that the germ cell will survive carrying sublethal mutations that can be transmitted to the embryo with fertilization.

TABLE 2. OUTCOME OF ADULT FEMALE EXPOSURE TO IRRADIATION

<u>Post-Exposure Mating Interval</u>	<u>(Wks)</u>	<u>Pregnancy Outcome</u>
(ova, 2° oocyte)	1	Reduced fertility, recessive (specific locus) mutations
(2° oocyte, Graafian follicles)	2	Dominant lethal and recessive (specific locus) mutations
" "	3	" "
" "	4	" "
" "	5	" "
" "	6	" "
(primordial follicles)	7	Reduced fertility, no mutations

Adapted from Russell, 1956.

In summary, it appears that the major outcome of radiation or chemical insult to the female germ cells is infertility. This can be permanent or reversible, depending on the number of oogonia or primary follicles destroyed and the age of the female at the time of exposure. Another possible consequence is transmission of mutation to the embryo, which could occur with fertilization of genetically damaged ova. In this case, the most likely outcome is early spontaneous abortion, insofar as all the cells of the embryo would carry the genetic damage.

EARLY SPONTANEOUS ABORTION AS AN INDICATOR OF GERM CELL DAMAGE

Concern about the effects of environmental exposure on the germ cells of men and women is increasing as evidence accumulates that genetic damage to the germ cells is strongly implicated in the occurrence of spontaneous abortion. Approximately 15% of recognized pregnancies end in spontaneous abortion, or early fetal loss, by 28 weeks gestation. The probability of spontaneous abortion is highest in early pregnancy, and gradually decreases with increasing gestational age (Table 3). Approximately 1/3 of spontaneous abortions occurring between 8-28 weeks of gestation are chromosomally abnormal. Many of the chromosomal anomalies found in abortuses are rarely or never found in term births (Table 4). Examples of embryolethal chromosomal anomalies are triploidies and tetraploidies, while other anomalies (trisomy 13, 18, and 21) are found in abortions and term births, but at higher levels in abortions. Chromosomal abnormalities are at least 60-fold more common among spontaneous abortions than term births (Report of Panel II, 1981).

TABLE 3. TEMPORAL INCIDENCE (%) OF FETAL LOSS

Duration of Pregnancy (Wks from LMP)	Probability of Fetal Loss*
4	10.8
8	7.0
12	4.5
16	1.3
20	0.8
24	0.3
28	0.3
32	0.3
36	0.4

* Probability that a spontaneous abortion will occur in the following 4 weeks.
Adapted from Schlesselman, 1979.

TABLE 4. INCIDENCE (PER 1000) OF SELECTED CHROMOSOMAL ANOMALIES

<u>Anomaly</u>	<u>Induced Abortions</u>	<u>Spontaneous Abortions</u>	<u>Livebirths</u>
Sex Chromosome			
45X	8.00	43.42	0.05
47XXX	6.00	0.00	0.52
47XXY	4.00	4.20	0.46
47XYY	2.00	0.00	0.46
All Trisomies	16.00	156.85	1.27
13	-----	9.80	0.05
18	2.00	7.00	0.12
21	-----	7.00	1.25
16	2.00	61.62	0.00
Triploid	2.00	50.42	0.00
Tetraploid	2.00	14.01	0.00
Rearrangements	0.00	12.61	2.37
Other	24.00	40.62	0.30
All Abnormal	68.00	322.13	6.20
Number Studied	500	714	56,952

(From Report of Panel II, p. 71)

Of the 2/3 of spontaneously aborted specimens that are chromosomally normal, about half have physical malformations. The highest frequency of morphologically abnormal conceptions occurs in the earliest abortions (<28 days), and gradually decreases with increasing gestational age (Table 5). Most of the early abortions consist of conceptuses with general growth disorganization most likely resulting from "blighted" ova or disruption early in gestation. Later abortions tend to contain fetuses with malformations in specific organ systems, suggesting the occurrence of teratogenesis. Normal

abortuses which lack chromosomal anomalies tend to have a high incidence of placental inflammation suggestive of uterine infections (Ornoy et al., 1981).

TABLE 5. INCIDENCE OF MORPHOLOGICALLY ABNORMAL ABORTUSES

	Group I (0-5mm) <u>< 28 Days</u>	Group II (6-30mm) <u>29-45 Days</u>	Group III (31-100mm) <u>46-89 Days</u>	Group IV (101+mm) <u>90-144 Days</u>
Number Examined	83	122	177	116
Number Abnormal	73	71	56	23
Percent Abnormal	88	58	32	20

(Report of Panel II, 1981)

It is generally believed that most chromosomal abnormalities in the conceptus arise in the germ cells of either parent prior to conception or at fertilization. Exposures that occur during pregnancy rarely, if ever, induce chromosomal abnormalities. Early spontaneous abortuses with general growth disorganization are also most likely due to germ cell damage prior to or during conception. Exposures that occur during pregnancy are usually associated with specific malformations, decreased birth weight and functional deficits, and not with chromosomal abnormalities. Table 6 contains information on the frequency of the most common types of reproductive dysfunction. As can be seen, infertility, spontaneous abortion, and chromosomal anomalies in spontaneous abortions are the most common types of reproductive failure in the human population. Given the prevalence of these outcomes, it seems particularly urgent that more experimental attention be given to identifying the mechanisms and the environmental agents associated with germ cell damage.

TABLE 6. FREQUENCY OF SELECTED REPRODUCTIVE ENDPOINTS

Event	Frequency Per 100	Unit
Failure to conceive after 1 year	10-15	Couples
Spontaneous abortion 8-28 weeks	10-20	Pregnancies or Women
Chromosomal anomalies in spontaneous abor- tions, 8-28 weeks	30-40	Spontaneous Abortion
Chromosomal anomalies from amniocentesis, >35 years	2	Amniocentesis Specimens
Stillbirths	2-4	Stillbirths & Livebirths
Birthweight <2500 grams	7	Livebirths
Birth defects	2-3	Livebirths
Chromosomal anomalies, livebirths	0.2	Livebirths
Severe mental retardation	0.4	Children to age 15 years

(Report of Panel II, 1981)

OUTCOMES OF PRENATAL EXPOSURE

The susceptibility of the conceptus to extrinsic insult is as complex and varied as the developmental process itself. Each of the major developmental stages, i.e., the pre-implantation, embryonic, fetal, and early postnatal periods possesses a characteristic

vulnerability. In the pre-implantation period, fertilization, blastulation, gastrulation, and early erosion of the uterine wall preceding implantation occur. This stage is characterized by susceptibility to embryolethality but rarely to teratogenicity. Insult during this period can result in repair through compensatory hyperplasia of relatively undifferentiated cells, or early embryonic death. This period occurs during the first three weeks of human development and the first six days of rodent gestation. The early spontaneous abortions discussed in the previous section could represent exposure of the embryo during the preimplantation period, but the majority are more likely to result from germ cell damage when chromosomal anomalies are involved.

During the embryonic period, organogenesis occurs from the third to eighth week in humans, and from 7 to 16 days in rodent gestation. The organogenesis period is characterized by the migration and association of cells and tissues into organ rudiments and is a period of particular vulnerability for induction of structural birth defects.

Histogenesis, functional maturation, and growth are the major processes occurring during the fetal period. Insult during this period leads to a broad spectrum of effects which can be generally manifested as growth retardation or specifically expressed as functional disorders or transplacental carcinogenesis. The fetus is more resistant to lethality than is the embryo, but the incidences of late fetal loss and stillbirths are measurable.

The occurrence of birth defects in newborns has been the most extensively studied outcome in reproductive studies. It is important to understand, however, that malformation in newborns is one of many possible outcomes resulting from insult to the reproductive system, and is relatively rare. It is possible that exposure to the same agent may cause a spectrum of effects, ranging from spontaneous abortion to malformation, to growth retardation, and to behavioral abnormalities. The outcomes may vary with dose and time of exposure, as well as with fetal and maternal factors. Of the adverse outcomes described so far, birth defects occur in most cases from exposure to the pregnant female, and not from exposures prior to conception or in early gestation.

In human studies, malformations are identified in stillbirths or newborns. For small populations characteristic of occupational and environmental studies, the rarity of most individual malformations (Table 7) necessitates grouping malformations by organ system or in major and minor categories.

TABLE 7. COMMON MAJOR MALFORMATIONS IN NEWBORN INFANTS

Malformation	Rate per 1000
Anencephaly	1.04
Myelomeningocele	0.33
Congenital hydrocephalus	0.36
Transposition of the great arteries	0.26
Ventricular septal defects	1.17
Hypoplastic left heart	0.23
Patent ductus arteriosus	0.72
Cleft palate	0.33
Cleft lip, cleft lip + cleft palate	0.78
Undescended testes	1.21
Hypospadias - first degree	2.12
Clubfoot	2.05
Talipes equinovarus	1.01
Metatarsus adductus	0.49
Congenital dislocation of the hip	0.78
Polydactyly - type B	2.15

(From Panel II Report, 1981.)

SUSCEPTIBILITY OF THE MALE TO REPRODUCTIVE IMPAIRMENT**MATURATION OF THE MALE REPRODUCTIVE SYSTEM**

In the early fetal period, prespermatogonial cells undergo mitotic proliferation as in the female. As the testes form, Sertoli cells and Leydig cells differentiate, active hormone secretion begins, and the basic pattern of the male reproductive tract is established. As a direct consequence of hormonal activity in the fetal testis, changes occur in the presumptive female (Mullerian) and male (Wolffian) duct systems which bring about the male pattern of development. The Mullerian duct system regresses, and the Wolffian ducts undergo growth and differentiation under the influence of testosterone production by the testes. The accessory glands and external genitalia of the male system are derived from the Wolffian ducts (Gondos, 1980).

A major difference in male and female germ cell development is that male germ cells do not enter meiosis until the time of puberty. Rather, in the late fetal/early neonatal period when oogonia enter into prophase I of meiosis, the prespermatogenic cells enter a period of mitotic arrest. Mitotic activity of prespermatogonial

cells is re-initiated at about 10 years of age, and active spermatogenesis begins some 3 years later (Hafez, 1977). This process begins with transformation of prespermatogonia to type A_S spermatogonia. These stem cells (A_S) are believed to occur singly and are randomly distributed throughout the length of the tubules. Their division results either in two separate A_S progeny cells that renew the stem cell compartment, or their division may be incomplete, such that paired progeny cells are joined by an intercellular bridge (spermatogonia A paired). These latter cells begin a series of synchronous divisions, and are transformed into A₁ spermatogonia which proceed to differentiate and undergo synchronous divisions giving rise to A₂, A₃, A₄, intermediate and B spermatogonia, which then enter meiosis to become primary spermatocytes (Oakberg and Huckins, 1976). This process is diagrammed in Figure 3.

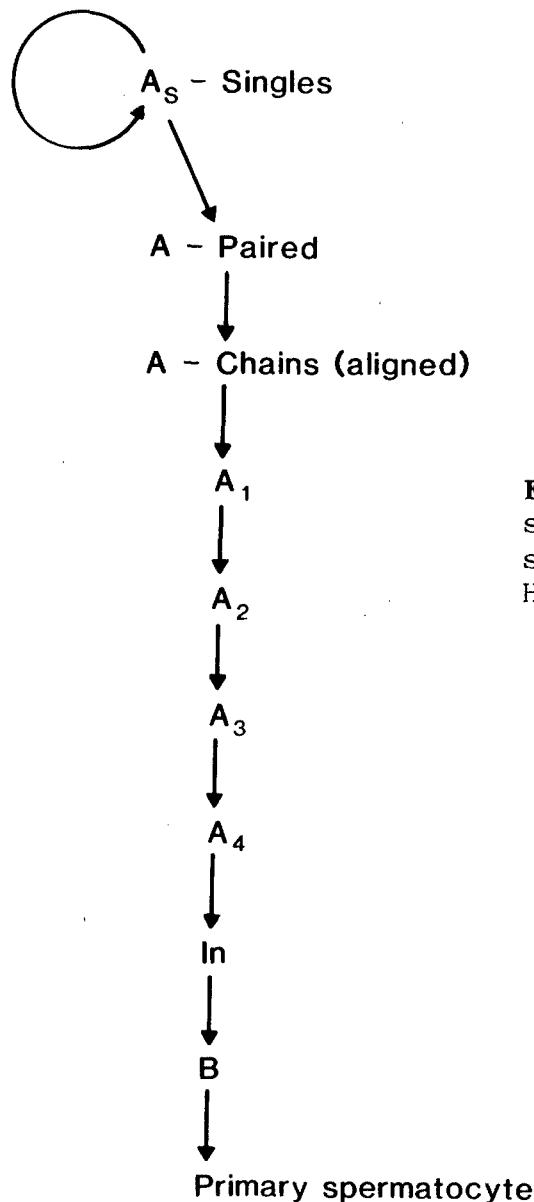


Figure 3. Differentiation of A_S spermatogonial stem cells to primary spermatocytes. From Oakberg and Huckins, 1976.

Spermatocytes undergo two meiotic divisions which are identical to those described for the female except that the progeny cells are of equal size, and extensive time intervals do not retard the progress of cells through to the spermatid stage. As in the female, a high percentage of germ cells degenerate during meiotic prophase. In humans, a cell loss of 36% occurs during the early spermatocyte to the spermatid stage due to physiologic atresia (Salisburg et al., 1977). Unlike the female, however, meiotic cell loss to atresia can be replaced by continual production of differentiated A_{pr} spermatogonial cells from mitotic divisions of the A_s stem cells. Thus, physiologic or drug-induced atresia of germ cells does not result in a shortening of the fertility span in the male, although it may cause temporary reduction in sperm count.

The function of the testis is controlled by at least two pituitary hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH stimulates Leydig cells to synthesize androgens, principally testosterone, which control the functional activity of accessory sex organs and development of secondary sexual characteristics. The role of FSH is not clear but it is generally recognized as acting on the Sertoli cell and as being important in the initiation of spermatogenesis at puberty and maintenance of optimal testicular function in the adult.

EFFECTS OF CHEMICAL AND PHYSICAL AGENTS ON SPERMATOGENESIS

Many more chemical and physical agents have been studied for their effects on spermatogenesis than for oogenesis. The number is so large that it is not reasonable to discuss individual compounds. Instead, a brief overview of the types of test systems available for studying male reproductive insult will be given so that an understanding of the array of adverse outcomes possible with chemical insult can be achieved.

The most frequently applied test for measuring male infertility and germ cell mutation in laboratory animals is the dominant lethal test. Males are treated with the test agent, and mated sequentially with control females, and females sacrificed at midpregnancy for measurement of embryolethality. Alterations in male fertility are determined by the ability of treated males to inseminate control females, while mutations in sperm are detected by an elevation in embryolethality in litters sired by treated males.

Female dominant lethal tests are not routinely performed because female germ cells are generally less sensitive to mutation induction than male germ cells. The increased sensitivity of male germ cells to dominant lethal or other types of mutations is based on the high cell division rate of stem cell spermatogonia. As indicated in Table 8, the stem cell spermatogonia of male mammals continue to replicate throughout the breeding lifespan, which

increases sensitivity to replication-dependent mutations. In female germ cells, mitosis ceases during the fetal stage, and the oocyte remains in a resting stage from birth until ovulation. In humans, oogonia pass through approximately 21 divisions per life-span, while spermatogonial stem cells pass through 380-540 divisions, depending on paternal age at conception. Therefore, as the oocyte has fewer divisions and spends such a high proportion of time in a nonreplicating state, the incidence of replication-dependent mutations will be lower than in the male (Lyon et al., 1979).

TABLE 8. CELL DIVISIONS IN FORMATION OF GAMETES

<u>Female</u>	<u>Humans</u>	<u>Mouse</u>
Divisions before formation of germ cells	?	10 - 13
Maximum no. of oocytes	3.4×10^6	1×10^4
Cell divisions in oogenesis	21	13
<u>Male</u>		
Divisions before formation of germ cells	?	10 - 13
Number of stem cells	5×10^8	1×10^6
Division cycle	16 days	8 days
Cell divisions to mature sperm	380 - 540 ^a	40 - 80

^a At ages 28-35 years
Adapted from Lyon et al., 1979

The dominant lethal test, as well as the heritable translocation and specific locus test are the methodologies most used in laboratory animal studies to assess the mutagenic potential of chemicals in male germ cells. The genetic risk evaluated is an increase in spontaneous abortion and genetically-based disease in the F₁ generation. Early spontaneous abortion, as previously discussed, can be used as an indicator of male germ cell mutation as well as of genetic alteration of female germ cells. It is usually not possible to distinguish whether early spontaneous abortion occurs as a result of male or female germ cell damage, unless one of the parents is differentially exposed to the toxic agent.

Other test methods exist to more specifically assess male infertility based on analysis of semen samples. Gross parameters measured include ejaculate volume, sperm concentration, percentage of motile cells, and sperm morphology. Parameters of sperm function that can be quantitatively assayed are sperm movement characteristics and in vitro penetration of hamster oocytes by sperm. In addition, special stains can be applied to measure the number of Y chromosomes in sperm from semen samples. The potential for subfertility or infertility is indicated when sperm concentration is reduced, motility depressed or altered, abnormal morphological forms elevated, or in vitro penetration of oocytes depressed. These parameters are considered more indicative of subfertility or infertility than of the potential for adverse pregnancy outcome because sperm with these altered characteristics are usually selected against in the female reproductive tract.

Thus, the major outcomes of insult to the male reproductive system are the same as those described for the female, i.e. infertility and early spontaneous abortion. The male appears to be somewhat more susceptible to induction of replication-dependent mutations in the germ cells, and less susceptible to infertility resulting from depletion of germ cells, than the female. In neither case is it likely that birth defects will occur from germ cell toxicity. Birth defects are rare outcomes that occur with exposure of the embryo at a specific stage of development. Many of these embryos abort shortly after exposure, with only a small fraction of the exposed population making it to term carrying malformations. Given that malformations represent the "tip-of-the-iceberg" of reproductive toxicity, examination of adult infertility and early spontaneous abortion would seem more relevant in studies of environmental and occupational exposure.

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WHOLE ANIMAL SYSTEMS FOR MEASURING CHEMICALLY INDUCED GENOTOXICITY

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One step in the initiation of carcinogenesis involves damage to the DNA. Mutations in germ cell DNA are responsible for true genetic changes. Numerous techniques are now available to assess the genotoxic activity of a chemical in a variety of bacterial or mammalian cell culture assays (Butterworth, 1979; Hollstein et al., 1979) such as chemically-induced mutagenesis (McCann et al., 1975; O'Neill et al., 1977), DNA damage and repair (Kohn, 1979; Swenberg and Petzold, 1979; Williams, 1976), cell transformation (Heidelberger, 1975; Krahn, 1979; Mishra et al., 1980) and sister chromatid exchange (Carrano et al., 1978; Wolff, 1977). These assays have been termed short-term tests for potential carcinogenicity because of a demonstrated correlation between carcinogenicity and activity in these assays for many classes of chemicals. Such systems are, however, severely limited in their predictive abilities because they do not reflect important species, strain, sex and organ specificities commonly observed in chemical carcinogenesis, and correlations retain a high degree of subjectivity.

Specificity in chemical carcinogenesis can be the result of differences in uptake, distribution, metabolism, DNA repair, detoxification, and excretion. For example, many procarcinogens must be metabolized to active forms that covalently bind to the DNA. Yet, the cells used for assessment of mutagenic activity are generally not metabolically competent. The metabolic activation system most commonly employed in bacterial and mammalian cell culture systems is a postmitochondrial supernatant (S9) from a rat liver homogenate. This is a crude preparation which is biased toward activation at the expense of detoxification reactions. In addition, important enzymes such as nitroreductases are either missing or nonfunctional in standard S9 preparations.

In assessing parameters such as sex specific chemical carcinogenesis or induction of genetic damage, it becomes imperative to employ assays which examine the action of chemicals in the whole animal. Several whole animal assays have been developed and an increasing amount of research is being devoted to this area

(Albertini, 1980; Petzold and Swenberg, 1978; Parodi et al., 1981; Mirsalis and Butterworth, 1980; Sega and Sotomayor, 1981; Wyrobek and Bruce, 1975; Fahrig, 1975; Brewen, 1977; Allen and Latt, 1976; Kligerman et al., 1981).

The value of whole animal systems is illustrated by research with nitroaromatic compounds in the *in vivo/in vitro* hepatocyte DNA repair assay (Mirsalis and Butterworth, 1980). This assay measures chemically-induced DNA repair in primary cultures of hepatocytes isolated from rats treated with suspect genotoxic agents. Following treatment by an appropriate route of exposure, primary cultures of hepatocytes are prepared and incubated with ^3H -thymidine. If the hepatocyte DNA is damaged in the animal such that the cells are undergoing excision repair, ^3H -thymidine will be incorporated into the DNA of the cultured cells. This unscheduled DNA synthesis (UDS) is quantitated by autoradiography and can clearly distinguish between cells in repair and cells in S-phase. The adult rat liver has a low frequency of cells in S-phase (~0.1%). The net number of silver grains over the nucleus (NG) is calculated by subtracting the grain count from the highest of three adjacent nuclear sized areas over the cytoplasm from the grain count over the nucleus. Scoring is performed with a computerized grain counter interfaced via a TV camera to the microscope.

Genotoxic hepatocarcinogens such as 2-acetylaminofluorene (AAF) and dimethylnitrosamine (DMN) have been shown to induce a response in this system while hepatotoxins such as CCl_4 do not (Table 1). The assay detects genotoxic hepatocarcinogens from a variety of chemical classes, including nitroaromatics, aromatic amines, direct acting agents, mycotoxins, nitrosamines, and azo compounds (Mirsalis et al., 1981b).

Technical grade dinitrotoluene (DNT), consisting of approximately 75% 2,4-DNT, 20% 2,6-DNT and lesser amounts of the other isomers, has been shown to be a potent hepatocarcinogen in rats (Chemical Industry Institute of Toxicology, 1978). After one year, male rats receiving 35 mg/kg/day DNT in the diet had a 100% incidence of hepatocellular carcinomas compared to 50% in the females and 0% in the controls. Yet, DNT produced no response in a variety of short-term tests for genotoxicity including cell transformation in 10T1/2 cells*, mutagenesis in CHO cells (Abernethy and Couch, 1981) and UDS in *in vitro* treated cultures of hepatocytes (Bermudez et al., 1979). These results raised the following questions: 1) Why was DNT negative in a variety of *in vitro* short-term tests?; 2) Was there an assay that would show activity with DNT?; and 3) Could such an assay reflect the differences in susceptibility between the male and female rats?

*Boreiko, C. J. (1971), Personal communication.

TABLE 1. INDUCTION OF DNA REPAIR IN HEPATOCYTES FROM RATS TREATED IN VIVO¹

Compound ²	Dose (mg/kg)	NG ³ \pm SE ⁴	Percent in Repair \pm S.D. ⁵
Corn Oil (control)	--	-4.4 \pm 0.5	0.3 \pm 0.8
Dimethylnitrosamine	10	22.2 \pm 1.9	78.0 \pm 6.0
2-Acetylaminofluorene	50	14.0 \pm 2.3	71.3 \pm 13.0
CCl ₄	100	-3.2 \pm 0.7	0.7 \pm 1.0

¹ Adapted from Mirsalis and Butterworth (1980).

² All compounds were administered by oral gavage two hours prior to hepatocyte preparation.

³ NG is the grains over the nucleus minus the grains in the highest of three nuclear sized areas adjacent to the nucleus in the cytoplasm.

⁴ Standard error (S.E.) represents slide to slide variability.

⁵ The percent in repair is defined as the percentage of cells with greater than or equal to 5 NG.

DNT produced a strong dose-related response in the in vivo/in vitro hepatocyte DNA repair assay at 12 hour post treatment (Mirsalis and Butterworth, 1981a) and in hepatic initiation-promotion protocols (Leonard and Popp, 1981). Further, at 48 hours post-treatment a 50 fold increase in cells in S-phase was observed. This combination of DNA damage followed by induced cell turnover greatly enhances the probability of producing mutagenic events and indicates that DNT is, indeed, genotoxic to the target cells.

A possible explanation for the discrepancy between the in vitro and whole animal systems is that metabolism by gut flora may be required to produce the proximate or ultimate genotoxic metabolites of DNT. To examine the role of gut flora on the genotoxicity of DNT, the degree of DNT-induced UDS was measured in axenic rats as well as rats containing the normal complement of gut flora. Extensive DNT-induced DNA repair was observed in rats with normal gut flora but not in rats lacking gut flora, confirming that metabolism of DNT by gut flora is an obligatory step in the genotoxicity of this and possibly other nitroaromatic compounds (Mirsalis et al., 1981a).

The response to DNT in the in vivo/in vitro hepatocyte UDS assay was compared in male and female rats (Mirsalis and Butterworth, 1981b). DNT produced far less UDS in the female relative to the male animals (Table 2). Similarly, the female rats exhibited only a fraction of the response in the induction of cells in S-phase by

DNT relative to the males (Table 3). These results are consistent with the observed lower susceptibility of female rats to the hepatocarcinogenic effects of DNT.

TABLE 2. INDUCTION OF UDS IN MALE AND FEMALE RATS FOLLOWING TREATMENT WITH DNT

<u>Chemical</u>	<u>Dose (mg/kg)</u>	<u>Sex (n)</u>	<u>N.G. ± S.E.</u>	<u>Percent In Repair</u>
Control	---	M (4)	-4.2 ± 0.4	2 ± 2
		F (3)	-3.7 ± 0.7	2 ± 0
tgDNT	100	M (4)	15.1 ± 1.6	80 ± 4
		F (3)	4.6 ± 1.9	49 ± 12

Male and female Fischer 344 rats were treated with tgDNT in corn oil 12 hours prior to sacrifice or with dimethylnitrosamine (DMN) in water 2 hours prior to sacrifice. Controls received corn oil. (n) is the number of rats treated. Standard errors (S.E.) shown represent variation among animals. Percent in repair is the percentage of cells with > 5 NG. Adapted from Mirsalis and Butterworth (1981b).

TABLE 3. INDUCTION OF DNA REPLICATION IN MALE AND FEMALE RATS FOLLOWING TREATMENT WITH DNT

<u>Chemical</u>	<u>Sex (n)</u>	<u>Percent in S-Phase ± S.E.</u>
Control	M (5)	0.08 ± 0.01
	F (3)	0.16 ± 0.08
tgDNT	M (3)	4.57 ± 0.22
	F (3)	0.60 ± 0.05

Rats were treated with 100 mg/kg DNT or corn oil 48 hours prior to sacrifice. 3 slides scored/rat: 2000 cells/slide. (n) is the number of rats treated. Standard errors (S.E.) shown represent variation among animals. Adapted from Mirsalis and Butterworth (1981b).

Comparative metabolism studies in rats show sex specific differences in the route of excretion and the metabolic profile of 2,4-DNT excreted metabolites (Rickert and Long, 1981; Bond et al., 1981). Taken together with the *in vivo/in vitro* hepatocyte DNA repair studies, these results illustrate the value of whole animal systems in predictive toxicology and in defining those mechanisms responsible for the greater degree of tumor induction by DNT in male relative to female rats.

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TOXICITY, GENDER, AND NEUROBEHAVIORAL RESPONSES

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In a recent textbook on the interaction of genotype and drugs on behavior, a chapter was devoted to sex differences since sex is such an outstanding inherited trait (Broadhurst, 1978). The chapter was six pages long. Most work on sex differences was described as incidental rather than deliberate, and that sex differences as a specific study objective were relatively rare in psychopharmacology. If there are only a few studies in the psychopharmacology literature, then one can imagine how sparse the literature is in the emerging discipline of neurologic or behavioral toxicology. There is, however, a substantial body of information concerning gender and the brain.

Beyond those hypothalamic and pituitary specializations that regulate reproduction, the gender-neurobiology literature leads one to the conclusion that although the brains of males and females are far more similar than they are different, there are also many subtle functional, anatomic, and physiologic differences. When neurobehavioral responses to chemicals differ significantly between the sexes, the difference is usually one of vector magnitude rather than vector direction. The following examples illustrate the fact that gender can influence neurobehavioral responses to chemicals, although the mechanisms involved are not necessarily neural. Most of the studies reported are on rats, which are known to have gender-related differences in hepatic metabolism for many chemicals.

The sex difference in oxidation of drugs by liver microsomes is due mainly to the higher binding capacity of cytochrome P-450 of male rats, although a slight sex difference in the activity of microsomal NADPH-linked electron transport is a contributing factor. This sex difference in drug metabolism by liver microsomes seems to be restricted to rats since a clearcut difference in drug metabolism has not been demonstrated in studies of mice, guinea pigs, hamsters, rabbits, dogs, monkeys or man (Kato, 1974). Indeed, Kato (1974) stated that one can predict with some certainty if a drug shows a clear sex difference in rats but not mice, these differences are related to the metabolism of the drug and this sex difference will

not occur in other animal species or man. However, if there are clear sex differences in the response in both rats and mice, then the difference may be related not only to the drug metabolism but also to differences in tissue sensitivity.

Unfortunately, the neurobehavioral toxicity literature is too sparse to determine the validity of Kato's mouse-rat prediction paradigm. Hepatic metabolism was found to be the key factor in a study on the interaction of a tricyclic compound, cyclobenzaprine (CBZ), and alcohol induced narcosis in mice (Messiha and Barnes, 1979). The data indicated that male mice were more sensitive to ethanol than female mice (5 g/kg IP caused about 115 minutes of narcosis in males versus about 44 minutes narcosis in females), and that addition of CBZ (5 mg/kg) maximally prolonged the ethanol narcosis in males but submaximally prolonged it in females. *In vitro* studies showed that CBZ inhibited endogenous rat liver alcohol dehydrogenase, and it was suggested that *in vivo* inhibition of this enzyme was the probable cause of the prolonged narcosis.

Alcohol may also have a more potent effect in men than in women (Taberner, 1980). In the absence of ethanol the men had significantly faster reaction times than the women, 280 ms versus 300 ms latencies on a task that required one to thumb press a switch in response to a red light. A half hour to an hour after ingestion of 0.76 ml/kg ethanol the reaction time of men was lengthened about 60 ms versus only 11 ms in women. This was a significant change in men but not in women, and at this time the latency was about 340 ms in men versus about 312 in women. At 90 minutes post-ingestion, however, the women's performance was significantly degraded so that they also had a response time of 340 ms. Blood ethanol levels were not significantly different in the men or women at 30 or 90 minutes. Sex differences in either ethanol absorption or distribution were assumed, but there is no evidence to support this supposition.

The irreversible anticholinesterase DFP causes greater decreases in body temperature and water consumption in male rats than in females (Overstreet et al., 1979). These effects were related to less inhibition of brain acetylcholinesterase (AChE) in female than in male animals. In contrast to the effects of DFP, female rats were more sensitive to the hypothermic effects of pilocarpine, a directly acting muscarinic agonist. Thus, it appeared that females had at least the same number or sensitivity of muscarinic receptors as male rats. Further experiments showed that females had less DFP uptake into the brain, which was then demonstrated to be an indirect effect due to the discovery that females had higher baseline levels of serum cholinesterase. Females are apparently less sensitive to DFP because more of it is bound peripherally, reducing the amount available for penetration into the brain.

Male and female mice appear to be differently sensitive to methylmercury (MeHg) (Tagashira et al., 1980). When groups of males and females were fed 50 ppm daily for 30 days, and were tested daily on a rotarod (a rotating rod similar to log rolling), the females had a precipitous drop in performance after the ninth day and the males had a slight decline after the thirteenth day. Female performance dropped to nearly zero by day 13, when overt signs of MeHg poisoning were evident. Males continued performing at a somewhat depressed level for several more days, even though by day 27 some of them had died. Performance of the male survivors was essentially normal. The MeHg content of the brain at the onset of neurologic signs was about 20 μ g/g in both sexes. In this instance, the source of the enhanced female sensitivity could be attributed to more rapid penetration of MeHg into the brain, since a diet containing 50 ppm MeHg for females caused brain Hg to increase at the same rate as a 100 ppm MeHg diet for males.

Long-term exposure to metaldehyde in the diet causes a dose-related development of posterior paralysis in female rats, with a latency period greater than 550 days (Verschuur et al., 1975). Fifty percent of females were affected on 5000 ppm metaldehyde, some were affected at 1000 ppm and none at 200 ppm. Although the presenting sign was neurologic, the cause was a fracture or luxation of thoracic vertebrae and subsequent compression of the spinal cord. No significant histologic damage to other organs was seen, although relative liver weight was increased at 5000 ppm. Reproductive performance was impaired at 5000 and 1000 ppm but not at 200 ppm. Pregnancy accentuates the occurrence, but the lesion can occur in nonpregnant females and in males. The mechanism of the fracture/luxation is unknown, and the spinal cord was not thought to be the target organ.

Spontaneous neuropathy is a concomitant of aging in rats as well as humans. In control rats in a 24-month toxicology study, males had significantly more peripheral neuropathy than females (Cotard-Bartley et al., 1981). At 6 and 12 months the sciatic nerves were either normal or only slightly affected, but at 18 months about 90% of the males and 60% of the females had Wallerian-type degeneration of the sciatic nerves. At 24 months all rats were affected, but the lesions were more severe in males. The authors suggested that this may be adequate reason for not using rats in long-term neurotoxicology studies. Although the incidence of neuropathy increases with age, it is not clear whether the sex-distribution is a primary response or secondary to factors such as body weight, especially since these rats were housed in wire-cages.

The studies mentioned thus far have shown male-female differences in behavioral toxicity that might reasonably be attributed to or enhanced by non-neural differences between the sexes. Misinterpretation of the data might be another category of effects. One

study reported a sex-dependent hypoactivity in pre- and postnatally lead-exposed rats (Verlandieri, 1979). It was suggested that there was an interaction between lead and environmental stress, in this case the blaring of a horn. What was clearly apparent was that normal female rats ran many more revolutions in an activity wheel than normal male rats. It was particularly interesting that when the horn sounded the females increased their activity while the males decreased theirs. This is an instance where normal male-female responses were diametrically opposite. In the lead-treated rats, however, both male and female activity was depressed and the loud noise had no effect on either sex. Thus, this was not a case of a gender specific response to a neurotoxicant.

The relationship between gender and radiation-induced performance decrement has been well-defined for the rat (Mickley, 1980). In this experiment the rats were able to avoid foot shock by jumping onto a shelf that was 10 cm higher than the floor. There was a 5 second interval between a warning click and the onset of shock. Performance was 100% before exposure to 10 krad high electron irradiation (about 1 minute exposure). Immediately after irradiation, performance dropped to about 5% for males and 35% for females. Recovery was faster in females, and both males and females had recovered to near 100% in 20 minutes. Castrated males and ovariectomized females behaved similarly, both having the severe male type immediate incapacitation; both also had a more rapid recovery than intact males (but not as rapid as females). Testosterone had no effect on the performance of gonadectomized rats, but systemic estradiol injections caused both castrated males and ovariectomized females to respond the same as intact females. To address the question of neural versus general systemic effects of estradiol, gonadectomized male and female rats were implanted with chronic bilateral intracranial cannulas. The cannulas either contained crystalline estradiol or cholesterol (sham control), and were implanted either into the nucleus preopticus medialis or the nucleus amygdaloidus. Only those rats with estradiol implanted into the nucleus preopticus medialis had performance profiles similar to intact females, clearly demonstrating that an interaction of estradiol with a specific locus in the brain was responsible for the less severe decrements that occurred in female or feminized male rats.

As an aside, these same radiation studies showed some interesting male-female differences in blood pressure, both pre- and postexposure to irradiation (Mickley, 1980). Preexposure systolic blood pressure was about 94 mm Hg in female rats and 124 mm Hg in male rats, but post-irradiation systolic pressure was about 106 mm Hg in both the males and females. This is another example of a toxic response going in opposite directions as a function of gender.

Nicotine, which has been used as a botanical insecticide, stimulates nicotinic acetylcholine receptors in autonomic ganglia, at neuromuscular junctions, and in some pathways in the central nervous system. At low doses it depresses exploratory activity of mice in "Y" maze tests (Hatchell and Collins, 1980). Three strains of mice were studied to determine the effect of genotype and sex as it relates to liver and brain nicotine content, and "Y" maze spontaneous motor activity. There was no consistent relationship between motor activity and either the rate of liver nicotine elimination or brain nicotine level. But, the females of all three strains demonstrated less sensitivity to nicotine's depressant effects than males. The authors concluded that although liver elimination differences may account for some of the observed behavioral effects, differences in brain nicotine level and brain sensitivity were more important.

There are many male-female differences in brain neurotransmitters in those areas responsible for reproductive functions and behaviors, but the following reports demonstrate gender-related differences in neurotransmitters elsewhere in the brain. In a rat study of receptor density and distribution for γ -aminobutyric acid (GABA), 5-hydroxytryptamine (5-HT) and dopamine, nonreproductive sexual dimorphism was found only for dopamine, in the amygdala, cerebral cortex and cerebellum (Karakiulakis et al., 1978). Another study found differences in either norepinephrine or dopamine concentrations in rat caudate, paraventricular and periventricular nuclei and in the median eminence. The catecholamine contents were higher in the male rats' brains (Crowley et al., 1978). A third study (Becker and Ramirez, 1981) demonstrated that *in vitro* slices of rat extrapyramidal nuclei (striatal) release dopamine and norepinephrine when treated with amphetamine. The stimulated release of dopamine varied with the estrous cycle in females, and was greatest during estrus, followed by diestrus I and II. Release was not significantly increased during proestrus. Stimulated norepinephrine release was also greater during estrus and diestrus II. Male rats showed significant stimulated release of both dopamine and norepinephrine, and the norepinephrine release was greater in males than in females during proestrus, estrus or diestrus I. This paper also referenced other studies that suggested that extrapyramidal disorders of humans may be improved by gonadal steroids, and that sex differences have been reported on the occurrence of drug-induced dyskinesia and Parkinsonism. The differences between sexes indicate that chemicals that interact with catecholamine neurotransmitters might well have gender-related differences in effects. The nuclei involved suggest motor activity, food and water intake and other mechanisms of body weight control might be particularly gender sensitive. Dieldrin appears to be an example of this type of neurotoxicity.

Dieldrin, which is one of the chlorinated cyclodiene insecticides, can cause hyperexcitability, muscle fasciculations and clonic-tonic convulsions in mammals. Male rats are more sensitive to dieldrin than female rats, and at severe neurotoxic doses for males (50 mg/kg), males had a 25% decrease in whole brain norepinephrine (Wagner and Greene, 1974). Norepinephrine content was not significantly changed in females. Dopamine and 5-hydroxytryptamine contents were not changed in either sex. When dieldrin was fed in the diet at 50 ppm for several weeks, several subcortical structures had an initial decline in norepinephrine content that returned to normal in subsequent weeks. Some changes also were noted in 5-hydroxytryptamine, but none with dopamine. In contrast to the acute exposures, there were no sex differences noted in the chronic exposures.

Drugs known to affect catecholamine neurotransmission include chlorpromazine, d-amphetamine and apomorphine. As might be expected, these also affect one gender more than another in some measures. Female rats normally devote more of their time to stereotyped behaviors, such as bursts of rearing, sniffing, self-grooming, and head-bobbing, than do male rats. Injections of amphetamine will increase this stereotyped behavior in males but not in females. The response is dose-related from 1 to 6 mg/kg, and at 3 to 4 mg/kg the males show the same amount of stereotyped behavior as the females. Kindling is a phenomenon where previous exposure to a drug increases sensitivity to subsequent exposure to that drug. Male rats, but not females, show a clear kindling response to amphetamine. Injections of the drug given 12 weeks after a one-week sensitizing series of injections caused significantly more stereotyped behavior than with the original injection series (Flemenbaum, 1979). In another study (Golub and Kornetsky, 1975), prenatal exposure of rats to chlorpromazine reduced female rats sensitivity at four months of age to challenge doses of chlorpromazine and pentobarbital. The task in this instance was fixed-interval (2 minutes) with condensed milk as the reinforcer, and the level of responding in the absence of challenge was the same as controls. But, after drug challenge the female rats that did not have in utero chlorpromazine treatment had a more depressed rate of responding than pretreated females. In utero preexposure had no effect on male level of responding. The effect was not limited to chlorpromazine challenge, but also occurred with the barbiturate.

Studies of functional brain asymmetry in humans indicate that, for most individuals, the left cerebral hemisphere is specialized for language as well as for the execution of learned manual activities. The right cerebral hemisphere appears to be less involved with speech but more critical than the left hemisphere for perception, construction, and recall of stimuli that are difficult to verbalize. This functional lateralization appears to be more pronounced in men than in women (McGlone, 1980) and poses interesting

questions for the toxicologist. If the average man and woman process information differently, then perhaps early neurotoxic manifestations would be different, also.

The preceding review is nearly devoid of examples of human data because virtually none were found, but the available animal data clearly indicate that in certain circumstances the nervous systems of males and females will react differently to toxic substances. However, the data also suggest that more often than not there will be no differences in male and female response, or the differences will be negligible. This implies that, more often than not, neurobehavioral data from both sexes should be combined. There is a tendency in toxicology to perform studies on both sexes and to keep the data separate. This may be reasonable if one knows from previous experience that gender differences exist. In the absence of that previous experience, separating neurobehavioral data according to sex may unnecessarily halve the sample size at a considerable cost in statistical power. A conservative approach, when uncertain of the gender sensitivity, would be to utilize factorial designs. The typical experiment with both sexes and four levels of treatment (control and three levels of exposure to the toxic substance) would be a 2×4 factorial design, and would allow one to sort out the effects of treatment and gender separately. In the absence of significant interaction of gender \times treatment, the power for detection of treatment would be enhanced by utilization of data from both sexes. In the presence of significant interaction, the experimenter would separate the data according to gender and perform regular ANOVA.

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OPEN FORUM

DR. SCHWETZ (Dow Chemical U.S.A.): Until we get some other questions from the audience, I would like to start with one for Dr. Lamartiniere. Are there differences in the amount of DDT or methoxychlor that cause an effect on sex differentiation versus the amount required to effect the development of MAO activity? What is the duration of each of those effects? Are they reversible and does one change at a different time than the other?

DR. LAMARTINIÈRE (University of Mississippi Medical Center): We have looked at the sexual behavior of these animals when they were 120 days of age and it is a permanent effect of methoxychlor. The effect of neonatal castration and neonatal administration of testosterone, E2 or DS, have been demonstrated by a number of laboratories previously to be permanent up to two years of age. In the case of DDT exposure, we've only measured DS. This was only measured for 120 days but I presume the effect is permanent. We followed hepatic enzymes through 120 days of age for monoamine oxidase and we have not looked beyond this. The data I presented were through 63 days but we did one experiment where we followed this effect through day 120. We didn't follow them longer than that simply from the standpoint of economics.

DR. TORKELSON (Dow Chemical U.S.A.): Dr. Butterworth, is anybody working with your system in organs other than the liver?

DR. BUTTERWORTH (Chemical Industry Institute of Toxicology): Yes, two post-doctorals and I are looking at other tissues. We're working with spermatocytes and we just started some studies with kidney tissue and we're planning to branch out beyond this but it gets very difficult. The only system that we have working well we're just beginning to work on is with hepatocytes. Spermatocyte tests are well underway but other tissues such as lung, trachea, and kidney are what we're planning on working on in the next couple of years.

COL. CARTER (Air Force Aerospace Medical Research Laboratory): Dr. Butterworth, are you planning to expand your studies to use inhalation as a method of exposure?

DR. BUTTERWORTH: Yes, we would like to. We think that it's very important to expose the animals by inhalation. We haven't done that yet, but we plan on doing it.

DR. KIESOW (Naval Medical Research Institute): Dr. Lamartiniere, do you have any information about the mechanism of enzyme activity control? Is it activation, reduction or inhibition? Are there any details known?

DR. LAMARTINIÈRE: Are you asking about the organizational effects?

DR. KIESOW: Yes.

DR. LAMARTINIERE (University of Mississippi Medical Center): I guess I should have but for the sake of brevity I didn't go into the two different types of mechanisms during my presentation. As biochemists, we normally think of hormone action in terms of activational effects; that is, induction or repression, activation or inhibition which is due to the direct presence of a hormone or an effector that could increase or decrease enzyme activity. The organizational effects are in contrast to that. The developmental effect occurs from the initial action of the chemical hormone or effector which is not present later in life. In the case of activational effects you have to have the hormone or the chemical present to exert a change. Organizational effects would not be activation or inhibition or induction or repression. Is that sufficient?

DR. KIESOW: Dr. Butterworth, are you using Dr. Williams' method to perfuse the liver?

DR. BUTTERWORTH: We use the procedure developed by Gary Williams to get the hepatocytes out of the liver into culture. This is a perfusion method with collagenase.

DR. KIESOW: Have you experienced any reversal of damages that were due to the perfusion per se?

DR. BUTTERWORTH: We have no way of telling what the perfusion technique is doing to the system. We just found that it works very well and we go ahead with it. It's possible that something in the perfusion method could be altering the response but we don't see any effect.

DR. CROCKER (University of California, Irvine): Dr. Butterworth, did you say that you had compared the repair response in the intact animal with that in the cells dispersed from the liver after it had a toxic exposure?

DR. BUTTERWORTH: Yes, after exposure to DNT.

DR. CROCKER: Did you find the same effect if you looked in the animal or intact organ following the induction of repair as you did in the dispersed cells?

DR. BUTTERWORTH: It depends. The hepatocyte culture picks up many carcinogens such as DMN all by itself but for key ones such as dinitrotoluene it does not. There are some discrepancies. Let me give you another example. . .

DR. CROCKER: No, that's all right, I wasn't trying to compare whether the *in vitro* treatment of the cells mimicked the *in vivo* treatment, but rather whether the finding of repair was equally

efficient in the liver *in situ* or the liver with its cells dispersed and cultured before labeling with tritiated thymidine. I'm assuming this would require that you would have labeled the animal with tritiated thymidine and looked at repair in the animal.

DR. BUTTERWORTH: No, we have not looked at the situation where we added the thymidine to the animal and then tried to look at repair so we can't compare the differences there. Actually, it becomes technically very difficult. The reason that we're labeling in culture is that every cell sees the same amount of thymidine and the labeling responses are very clean and reproducible. When you put it in the animal, you have the same problems in that there are differences in metabolism, distribution, uptake of the thymidine, some portions are labeled very well, others not at all. So we find that by treating the whole animal and then pulling the cells out and labeling in culture, we have the best of both worlds. You get the toxicity in the whole animal but the labeling is uniform and reproducible with the cells in culture. We tried it both ways and found that this approach seemed to work better. One other point is that we do miss some carcinogens that are not hepatocellular carcinogens using this technique. For example, benzo(a)pyrene is negative in our system but it does not produce liver tumors. We would probably miss the tumorigenicity of chemicals that were specific for other organs and that's, of course, a disadvantage depending on how you look at it.

DR. RASMUSSEN (University of California, Irvine): Dr. Butterworth, I noted in some of your results you indicated that less than 100% of the cells showed unscheduled DNA synthesis. Have you been able to identify the cell types which are competent for unscheduled synthesis and those that are not?

DR. BUTTERWORTH: That is interesting. When we score the percentage in repair any cell with greater than 5 grains over the nucleus we would call in repair. And very often we'll see the situation where half of the cells will be in repair and half are not. We have no idea why that is. But if you increase the dose you see increases both in the total response and the number in repair. This once in a while begins to diverge. Say if you treat an animal and then wait for two days; look at them very often, you'll see say 10% of the cells which are heavily in repair and 90% which are all fixed up. So there is some divergence there, but we're not sure what that means.

DR. STEELE (National Institute of Environmental Health Sciences): Dr. Manson, did you see any age related differences in terms of an abnormal fetus when the females were X-irradiated or received a certain chemical?

DR. MANSON (University of Cincinnati College of Medicine): As far as X-ray is concerned, there is an interesting trend. Even women who receive therapeutic doses of pelvic X-irradiation who are close to the age of menopause tend to become permanently amenorrheic or in fact, to be precipitated into menopause whereas younger women 35 years of age and under tend rather to have one or two missed menstrual periods and then seem to recover and go on. The older a woman is, the more likely she is to run into premature onset of menopause even with therapeutic pelvic X-irradiation. And of course, there is a tremendously potent effect of maternal age on the incidence of Down's Syndrome which is irrevocable. It's a very obvious effect which has no counterpart in the male. There have been attempts to indicate that paternal age is associated but at best it's very weakly associated with the incidence of Down's Syndrome. In terms of general spontaneous chromosomal anomalies occurring in the offspring as a result of age or X-irradiation induced abnormalities, increased maternal age is clearly a predisposing factor. And this really doesn't seem to be the case with the male. Perhaps because there are frequent spermatogonial divisions providing fresh populations of sperm cells whereas in the female the oocytes continue to age from the time of birth. Maternal aging is a very heavily contributing factor, with the most notable cases being Down's Syndrome and then general amenorrhea and chromosomal aberrations with X-irradiation.

DR. SCHWETZ (Dow Chemical U.S.A.): I have one other question that can probably be answered by either or both of our behavioral experts and it has to do with agents that are delivered during pregnancy so that you have exposure of the developing embryo in utero; there's an increasing body of literature now on the effect of that type of exposure on the behavior of offspring after normal delivery. Are there examples of chemical or virus induced stress insult where there is an effect that is more apparent in either male or female, one more than the other in the offspring?

DR. LAMARTINIERE (University of Mississippi Medical Center): Do you mean in humans or in animals?

DR. SCHWETZ: In animals.

DR. MATTSSON (Dow Chemical U.S.A.): I can think of one reference and it's on a study in rats. It concerned exposing pregnant female rats to chlorpromazine. The performance decrement effects of chlorpromazine are the inhibition of motor behavior of rats at virtually any postnatal age. In rats that had been exposed prenatally to chlorpromazine there is a clear gender difference between the sensitivity of the males versus the females to doses of chlorpromazine when given to them as adults. My recollection is unclear if it's the male or female that was tolerant to the drug, but chlorpromazine given during pregnancy can affect the offspring when they are adults to the depressant effects of the drug at that time. The part of that article that stuck in my mind is that the depressant effects

of one of the barbiturates, and I don't remember which one for sure, was also changed in that the same gender that now was tolerant to the chlorpromazine was also tolerant to the barbiturate so it wasn't a chlorpromazine specific effect. They weren't able to identify whether this was changed hepatic metabolism or neurosensitivity or what, but it was a case of gender related prenatal effect demonstrated in the adult.

DR. LAMARTINIERE (University of Mississippi Medical Center): I would like to elaborate on that particular aspect. Miller showed that there is a higher incidence of liver cancer in male rats than female rats fed aromatic amines (AFF) and then this was carried a bit further by Weisberger et al. where they treated female rats during the neonatal period with estradiol and demonstrated that these animals when later exposed to AFF had a higher incidence of cancer. These studies suggest that neonatal exposure to estrogen is responsible for causing organizational effects perhaps by altering endocrine secretion to predispose for a higher cancer incidence in males. At a cancer symposium that was held in Raleigh, North Carolina last year, Dr. Jerry Rice presented some data on humans where there was a higher incidence for liver carcinoma in males than in females. I don't know if one can make a direct correlation between these responses, but perhaps they are due to the same type of organizational effects.

DR. MATTSSON (Dow Chemical U.S.A.): I just thought of another example which is methylmercury. Data from a recent study at Dow in which the dams were exposed to just a minimally toxic dose of methylmercury, female offspring did not gain weight as readily and there was evidence of performance decrement in learning to run a maze and the speed with which they would go through the maze and the effects were extremely significant. When such effects were noticed they were almost always in the females and it was always a decrement in performance. It appeared to be gender specific and the female offspring were more clearly affected than the males to prenatal maternal exposure to methylmercury. So there are instances, chlorpromazine and methylmercury. I guess what we're saying now with two diverse chemicals, one a heavy metal and the other one pharmaceutical, delayed manifestations were demonstrated to be more serious in one gender than the other.

SESSION III

THE APPLICATION OF TOXICOLOGY DATA TO HAZARD ASSESSMENT

Chairman

**Gale D. Taylor, Col., USAF, BSC
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AN OVERVIEW

Gale D. Taylor, Colonel, USAF, BSC

Toxic Hazards Division
Air Force Aerospace Medical Research Laboratory
Wright-Patterson Air Force Base, Ohio

This morning's session marks a distinct departure from the normal format of the annual Toxicology Conference. In this Conference we have, historically and quite appropriately, been primarily concerned with the information developed within the four walls of the toxicology laboratory. For many reasons, those of us employed in DOD R&D efforts must broaden our horizons. If for no other reason, we are obligated to insure that the resources allocated to our programs are applied to those efforts that most significantly support the mission of our respective military services. I do not believe that the military toxicologist can fulfill that obligation without an intimate appreciation for the beneficial uses of new materials, with little understanding of the military industrial work environment or of the current occupational health programs, and without a fair degree of empathy for those individuals who must seek to blend engineering controls, protective equipment, sampling strategies, and analytical techniques into a supportable and defensible industrial hygiene program.

Conversely, it is equally important for the industrial hygienist to spend some time walking in the toxicologist's moccasins. There is simply no way for the toxicologist to provide an all-inclusive cookbook for the industrial hygienist to use in every conceivable situation.

I have spent a large part of the past 20 years in various positions both in R&D and in the Occupational and Environmental Health Laboratory and believe that I have an excellent perception of the exceedingly complex problems facing both groups in the present occupational environment. Please understand that I don't profess to have many answers or solutions, just great empathy for both groups of professionals.

Perhaps the single largest concern that I have in this area can be directly traced to the absence of a coordinated approach for addressing health and environmental issues. It would seem that the mounting list of mutually inclusive problems confronting

toxicologists and industrial hygienists over the past several years should have served to bring the two groups closer together. Unfortunately, the opposite seems to be true. And, in my opinion, it's true not only in the military but also in industry, academia, and in professional societies as well. Far too often each group finds itself working a multifaceted problem alone and without benefit of the insight and expertise that resides in the other camp. I don't believe that today's session will solve all the problems of unsatisfactory coordination and communication, but I am hopeful that this session and the opportunities available this week to discuss mutual problems will serve as an initial step in permanently closing this gulf.

Although, as I've indicated, there is a multitude of problems requiring joint attention, there are three issues that should be singled out as priority items. First is the necessity for establishing a standardized occupational health program that will permit tracking an employee across a working lifetime with a reasonably accurate exposure history and a feedback mechanism to provide this information to the research community. Major Lombard will discuss a proposed program and its potential value to the R&D scientist later in this session.

The second problem, and one that I hesitate to even mention this morning because of the inherent overriding emotional and political considerations, is that of women in the workplace. I strongly support, as I'm sure do most of us in this room, the rights of all people to compete for jobs without discrimination of any kind. Yet this very openness of employment poses grave scientific and moral difficulties in addition to the purely legal problems. How do we protect the unborn? Do we totally disregard this uninvited visitor to the workplace? I think not, but do we establish controls and programs based on the possibility of fetal effect regardless of cost, inefficiency, etc.? Some see a policy slowly emerging through the courts. I do not. I believe that policies concerning this issue will evolve from the workplace and from concerned management. Those policies will, in turn, be established through decisions made by industrial hygienists and toxicologists, hopefully working together.

Finally, the last of the problems I want to touch on this morning concerns the gray area that exists between the toxicology laboratory and the human work environment. We in the laboratory can take a new material and study it in exhaustive detail. We can describe with great accuracy its physical properties. Given enough time and money, we can define its absorption, distribution, metabolism and excretion patterns. We can, with our superb analytical equipment and techniques, detect quantities of material at the part per trillion and even part per quadrillion levels. We can, with great precision and accuracy, determine concentrations of the material that produce no detectable toxic effects with single exposures, multiple exposures, continuous exposures, or

even across generations of exposures. Yet all this vast store of knowledge is based on subhuman animal models, and there are those individuals who deny the validity of any data derived from animal exposures. Isn't it strange that after all these years of comparative toxicology and after all the successes that can be identified in successfully transferring knowledge from the laboratory to the workplace that we still find ourselves defending the basic philosophy of toxicology time after time? In my more pessimistic moods, I believe I hear the arguments against comparative toxicology growing louder and gaining support. Somehow we have not presented our case properly. Perhaps it is our own conservatism in transferring animal derived data to human exposures that has been our own worst enemy. For, as I mentioned earlier, although we can precisely define acceptable exposure levels for experimental animals, we all become ultraconservative when we start establishing standards for the workplace. There are many admitted uncertainties. Perhaps we have not studied the most sensitive species, perhaps man physiologically handles the compound in a totally unique manner, perhaps any one of a dozen other variables. So we take the path of least resistance and start diminishing our toxicology base by safety factors that make us feel more comfortable but, in turn, erode the credibility of our findings. Then, far too frequently we saddle our industrial hygienists with workplace standards that are based on sampling and analytical sensitivities rather than on our voluminous toxicology data base. One frequently wonders why we take the time and expense for doing more than cursory screening when the lowest possible detectable level is more frequently used in standard setting than is a scientifically sound no-effect level.

Being an eternal optimist, however, I do see some movement back toward realistic standard setting. The efforts gaining momentum in many laboratories, including our own, in the sub-specialty of toxicokinetics will help bridge the abyss between laboratory and workplace. The comprehensive occupational health program conceived by the OEHL would be a valuable asset in this effort. We must recognize, however, that any movement toward more realistic standards inherently places increasing levels of responsibility on our industrial hygienists and on the surveillance programs they direct. It thus becomes extremely important for our colleagues in the OEHL to understand and actively participate in our efforts.

For all these reasons, I welcome the opportunity to chair this initial industrial hygiene session in the Conference and look forward to a productive and stimulating exchange of ideas.

**AN INDUSTRIAL HYGIENIST'S VIEW:
RELATIONSHIPS BETWEEN INDUSTRIAL HYGIENE SAMPLING,
HUMAN EXPOSURE STANDARDS, AND EXPERIMENTAL TOXICOLOGY**

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INTRODUCTION

One industrial hygiene specialty is the evaluation of hazards posed by small concentrations of toxic materials in the workplace air. A variety of instruments are used to measure the average concentration over specified periods of time. These measured concentrations are then compared with exposure standards to judge the quality of the workplace. Much has been written about proper statistical sampling strategies to insure that workplaces meet established exposure standards (Leidel, 1977; Tuggle, 1981). Much less has been written about whether or not exposure standards are created in such a fashion that a workplace which is in compliance is in fact as safe and healthful as desired (Zielhuis, 1979). This is an interdisciplinary question lying between industrial hygiene and environmental toxicology. It is the purpose of this paper to provide the environmental toxicology community some food for thought from the perspective of one member of the industrial hygiene community.

Throughout the discussion it is assumed that the only occupational exposure is through inhalation. If there are other routes of entry due to workplace design, then the kinds of standards discussed here are inappropriate. It is the U.S. Air Force policy to provide workplaces which meet the more stringent of the standards set by the Occupational Safety and Health Administration (OSHA Standards) or those set by the American Conference of Governmental Industrial Hygienists (ACGIH Threshold Limit Values, called TLVs). Because the source of the standard is not germane to this discussion, standards are often referred to by the generic term, Permissible Exposure Limit (PEL).

Many substances of interest to the USAF are regulated by two separate standards, a short one with a specified observation period less than 30 minutes and a long one with an observation period of 8 hours or more. From an industrial hygienist's perspective, this naturally leads to the question, "What are the consequences of two standards for a single substance?". From an environmental toxicologist's point of view the question might be phrased, "What data are required to justify setting two standards for a single substance?".

THE CONSEQUENCES OF DUAL PERMISSIBLE EXPOSURE LIMITS

To understand the consequences of dual PELs, it is first necessary to be familiar with the consequences of making decisions on the basis of a single PEL, and second to thoroughly understand some subtleties of measuring occupational exposures.

THE NATURE OF OCCUPATIONAL EXPOSURES

Occupational exposures can be viewed as the sum of an average exposure and a randomly varying exposure (Saltzman, 1970). The industrial hygiene sampling process averages out the random fluctuations during the observation period and reports the average exposure during that period. Because random fluctuations occur over long periods of time as well as over short periods of time, the measured exposure is seldom exactly equal to the true long-term average exposure. Many authors have reported that measured exposures tend to be lognormally distributed (Bencala, 1976; Leidel, 1977). This is a very helpful observation, since it means that a relatively simple mathematical model can be used to aid our thinking about the industrial hygiene decision process (Rock, 1981a and 1982a).

The model has two primary features, a normalized exposure variable, x , and a lognormal probability density function, $pdf(x)$. The use of a normalized exposure allows the model to apply to any occupational hazard. One merely divides the actual 8-hour time weighted average (TWA) exposure by the applicable permissible exposure limit (PEL) to create this normalized exposure ($x=TWA/PEL$). Note that whenever $x > 1.0$, the daily exposure exceeded the standard. It is not certain that occupational exposures are lognormally distributed, but they are certainly represented by a skewed distribution with properties similar to those of the lognormal distribution; there are no negative exposures, most exposures are small, and there are occasional large exposures (Hardis, 1982).

The great advantage of the lognormal exposure model is that each employee's work environment can be completely described by two parameters. The geometric standard deviation (GSD), which measures the variability of daily exposures, is one of these parameters. Any of the common industrial hygiene estimates of the cleanliness of a work environment may be used for the other parameter: GM, \bar{x} , e , or MPE. The median exposure is the geometric mean (GM); the long-term average exposure is the arithmetic average, \bar{x} ; the fraction of exposures which exceed the standard equals the probability that an exposure exceeds the standard, $e = P(x > 1)$; and the maximum probable exposure, MPE, is defined for purposes of this paper as that exposure which we expect to be exceeded one workday per year, $P(x > \text{MPE}) = 1/250$ (Gumbel, 1958, pg. 82). Recall that a typical working year includes 250 workdays (Rock, 1981a).

Figure 1 shows the distribution of exposures in nine different workplaces and illustrates the behavior of the long-term average exposure as a function of e and GSD (Rock, 1981a). Values of e close to zero correspond to relatively clean workplaces (only a small fraction of exposures exceed the standard) while values of e near one correspond to dirty workplaces (nearly all exposures exceed the standard). Values of GSD near one correspond to very stable workplaces (where all exposures are approximately equal), while values of GSD greater than 2.5 correspond to work environments similar to those experienced by firefighters or emergency maintenance workers (where both very large and very small exposures are common). The primary purpose of Figure 1 is to clarify and emphasize that each pair of values (e , GSD) specify a unique workplace with a well-defined distribution of exposures. The secondary purpose is to show that the long-term average exposure, \bar{x} , is a function of both e and GSD, and to provide a frame of reference for other figures.

Figure 2 is a contour plot of the long-term average exposure as a function of e and GSD. The nine "+" signs mark the locations of the nine sample workplaces plotted in Figure 1. Each point in the e , GSD plane represents one unique workplace, and the value of its long-term average exposure can be determined by interpolation between the contours. Each contour is labeled with the value of \bar{x} . We should all be able to agree that any workplace where the long-term average exposure exceeds the standard is unacceptable. This region is shaded with diagonal lines. The heavy contour where $\bar{x} = 95\%$ of the standard is labeled the average exposure limit (AEL). This is an arbitrary selection which may be applicable for some kinds of occupational exposures. It seems easier to find agreement on clearly unacceptable workplaces than on acceptable workplaces, so rather than suggesting that all workplaces in the horizontally shaded area are acceptable, it seems more reasonable to define all other workplaces as unacceptable. Note that these criteria reject three of the nine workplaces from Figure 1.

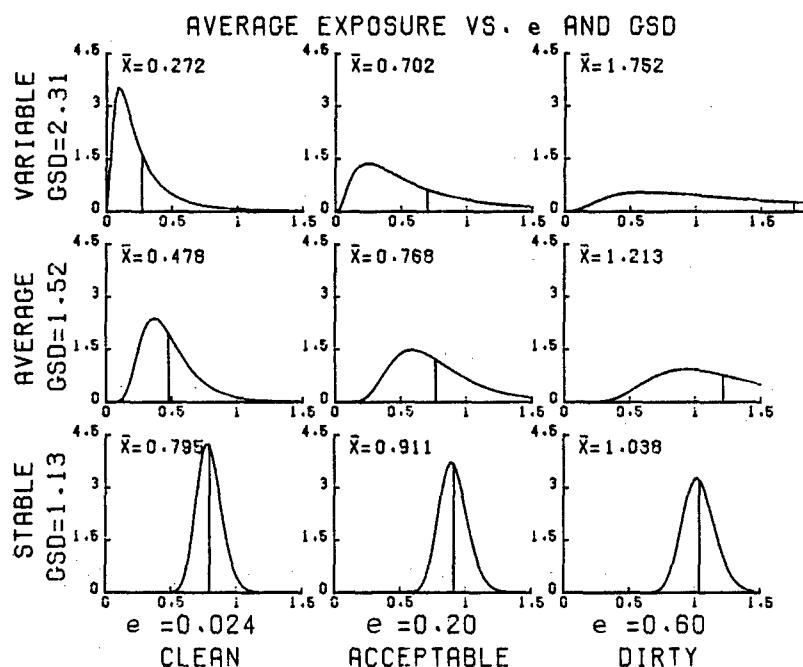


Figure 1. Probability density function for nine different workplaces showing \bar{x} , the long-term average exposure as a function of e , the fraction of exposures which exceed the standard and GSD, the geometric standard deviation of the exposures.

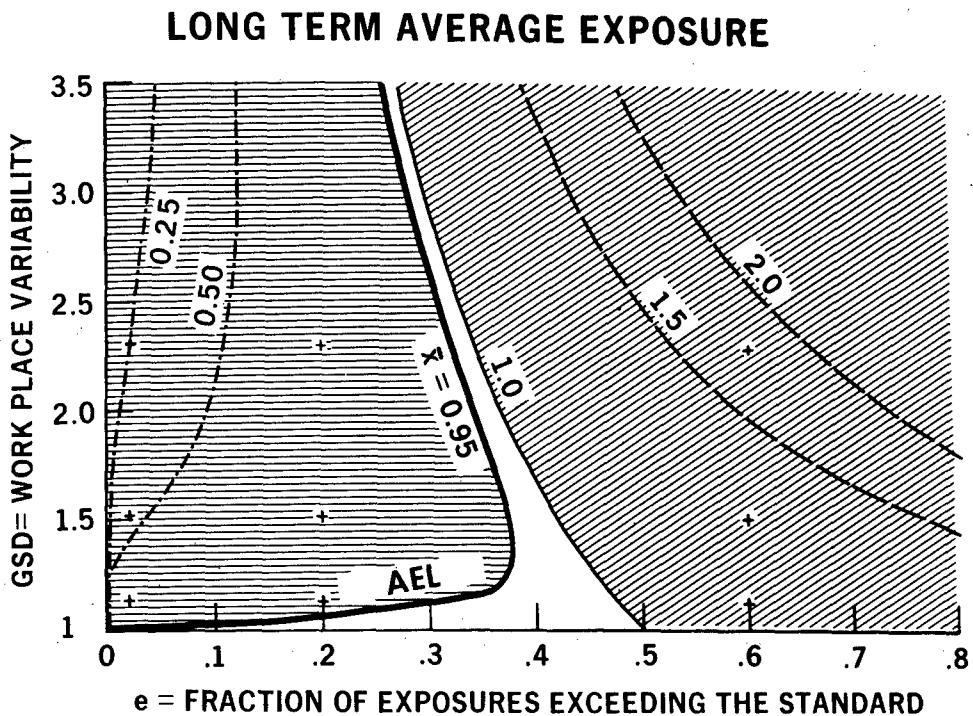


Figure 2. Contour plot of \bar{x} , the long-term average exposure, as a function of e and GSD. Each "+" marks the location of one of the workplaces from Figure 1.

Figure 3 shows the contours for the maximum probable exposure (MPE). The old ACGIH excursion factors suggested that a transient peak exposure during a workshift should never exceed 3 times the TLV, in the least dangerous situations. Therefore, there should be no objection to the concept that all workplaces with an MPE greater than 4 times the standard will be considered as unacceptable, especially since the MPE refers to a full period measurement rather than to an excursion which occurs during a period when the average exposure is less than the standard. There are some workplaces where a more stringent criterion is required, but none where a less stringent criterion would be acceptable. Note that the MPE exceeds 4 in only two of the nine workplaces (marked by "+") from Figure 1.

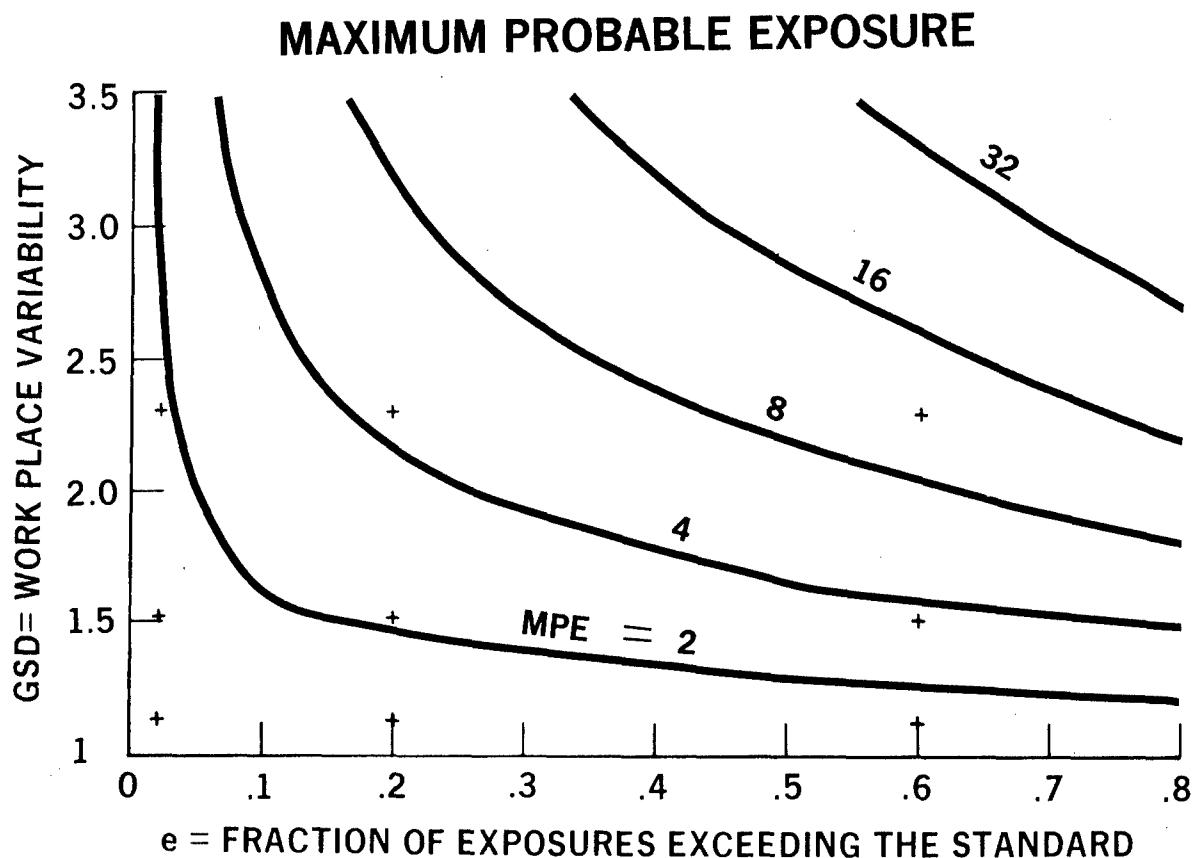


Figure 3. Contour plot of MPE, the maximum probable exposure, as a function of e and GSD. Each "+" marks the location of one of the workplaces from Figure 1.

Figure 4 shows the workplaces which are rejected in either Figure 2 or Figure 3. Engineering controls for an occupational hazard should never be designed to permit a workplace exposure distribution lying in this region. Figure 4 serves as an absolute frame of reference. It has been used to judge the quality of decisions made by various decision strategies (Rock, 1981c).

CLEARLY UNACCEPTABLE WORKPLACES

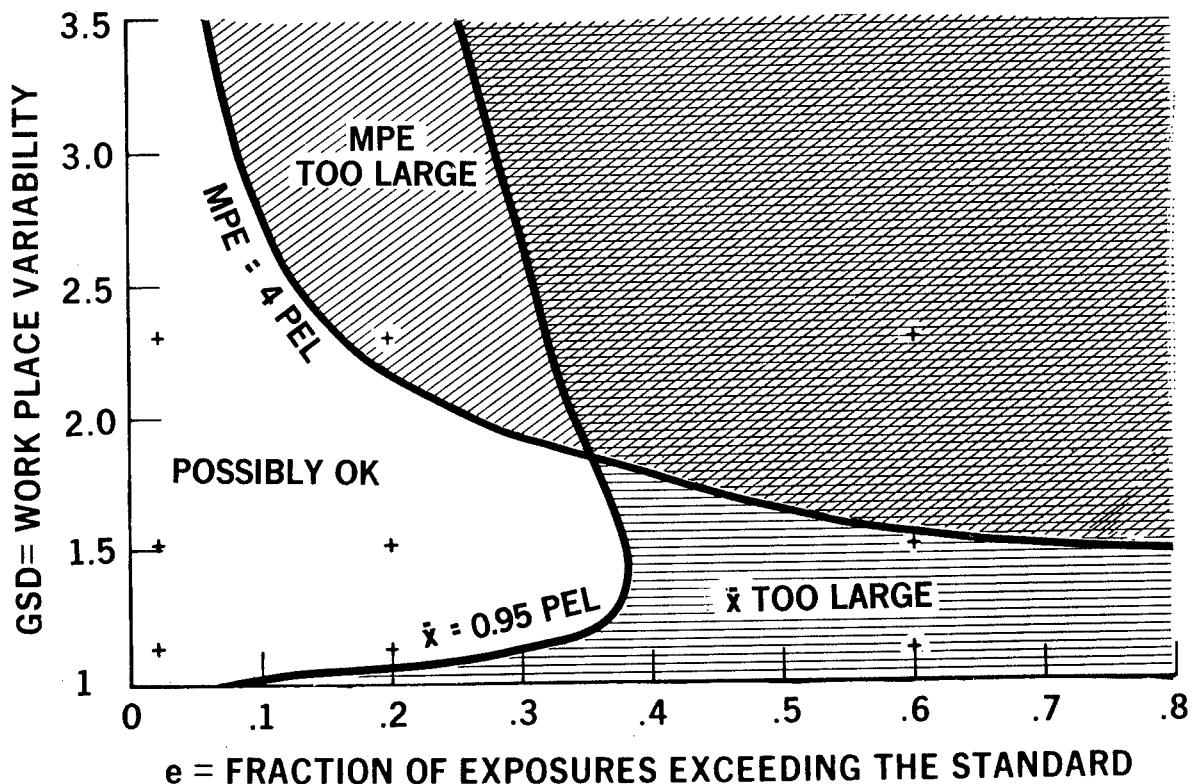


Figure 4. Contours showing workplaces which are clearly unacceptable, either because the daily maximum probable exposure is greater than four times the permissible exposure limit, or because the long-term average exposure is greater than 95% of the permissible exposure limit.

THE NATURE OF OCCUPATIONAL EXPOSURE MEASUREMENTS

Before proceeding further, a warning is in order. Real measurements are only estimates of exposures (LeClare, 1969). The uncertainties of the environmental sampling and analytical chemistry processes cause the distribution of real measurements to be broader than the distribution of ideal measurements (Leidel, 1975; Bar-Shalom, 1976). Although it is not often addressed,

even ideal measurements taken with perfect sampling and analytical processes exhibit a distribution which is significantly different from the distribution of exposures. This difference is one of the important factors to be considered in the standard setting process.

IDEAL MEASUREMENTS

In Figures 1 through 4 each workplace was described by two parameters, one to describe the variability of daily exposures (GSD) and the other to describe workplace dirtiness (e). In practice, the location of a workplace in the (e ,GSD) plane can be determined from a set of measurements of that workplace, all taken for the same observation period. If measurements are taken for a longer (shorter) observation period, then more (less) of the intrinsic environmental variability is averaged out by the measurement process. Thus, the variability of any set of measurements is inversely related to the length of the observation period. In contrast, the long-term average exposure is an intrinsic property of the workplace and is therefore independent of the averaging time (Roach, 1966; Saltzman, 1970). Both e and GSD are functions of the measurement process. Therefore, a single workplace can appear at different locations in the (e ,GSD) plane depending upon the averaging time used in measuring airborne contaminant concentrations.

Figures 5 through 7 illustrate some properties of measurements taken in a hypothetical workplace where the 2000-hour annual average exposure is assumed to equal half of the appropriate permissible exposure limit. Figure 5 shows the GSD of sets of possible overlapping measurements as a function of the averaging time used for the observations. It is based on an assumed 2000-hour working year as the maximum observation period and uses empirical equations derived from ambient air quality data (Larsen, 1971). Figures 6 and 7 show other characteristics of sets of measurements made in this sample workplace as a function of the averaging time used for these measurements. It is assumed that the measurements are nonoverlapping and that a complete set of measurements would last for the entire 2000-hour working year. Thus, a complete set would contain 2000 1-hour measurements or 250 8-hour measurements or 50 40-hour measurements. Figures 6 and 7 are discussed in more detail later in the text. First, consider two motivational examples designed to illustrate consequences of using currently available permissible exposure limits.

As a first example of conducting industrial hygiene measurements in this sample workplace, assume that formaldehyde is the contaminant of interest. Formaldehyde is regulated by a TLV of 2 ppm and carries the ceiling or C notation. To conform to the

conditions of Figures 5 through 7, assume that the one year average exposure level is 1 ppm. The GSD = 2.5 for 15 minute measurements, the GSD > 2.5 for all measurement sets taken with averaging times shorter than 15 minutes, and the GSD < 2.5 for all sets of measurements taken with averaging times longer than 15 minutes. Since this workplace has a long-term average exposure equal to half the standard, it will always be located in the (e, GSD) plane along the $\bar{x} = 0.5$ contour drawn in Figure 2. Comparing Figure 2 with Figure 4 reveals that whenever the GSD of the measurements exceeds 2.5, this workplace will be located in the region we have described as definitely unacceptable for 8-hour measurements. The ceiling standard makes Figure 2 valid for all averaging times. This workplace will be plotted in the clearly unacceptable region of Figure 2 by ideal measurements collected with a sampling method which has adequate sensitivity with averaging times shorter than 15 minutes, but will be plotted in the probably acceptable region by sampling methods which require longer sampling times. This example illustrates a practical deficiency of ceiling standards: given enough samples of short enough duration in most workplaces, you will eventually violate a ceiling standard.

MEASUREMENT GSD vs AVERAGING TIME

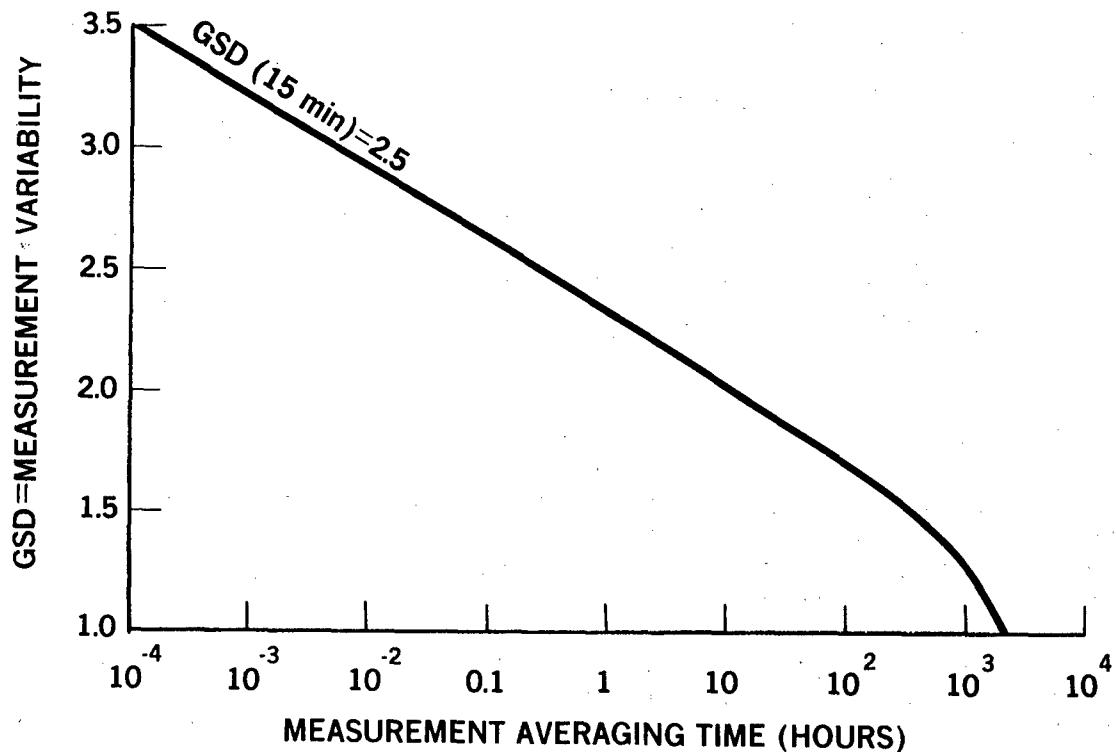


Figure 5. An illustration of the variability of a hypothetical workplace as a function of the measurement averaging time.

MAXIMUM ANNUAL MEASUREMENT

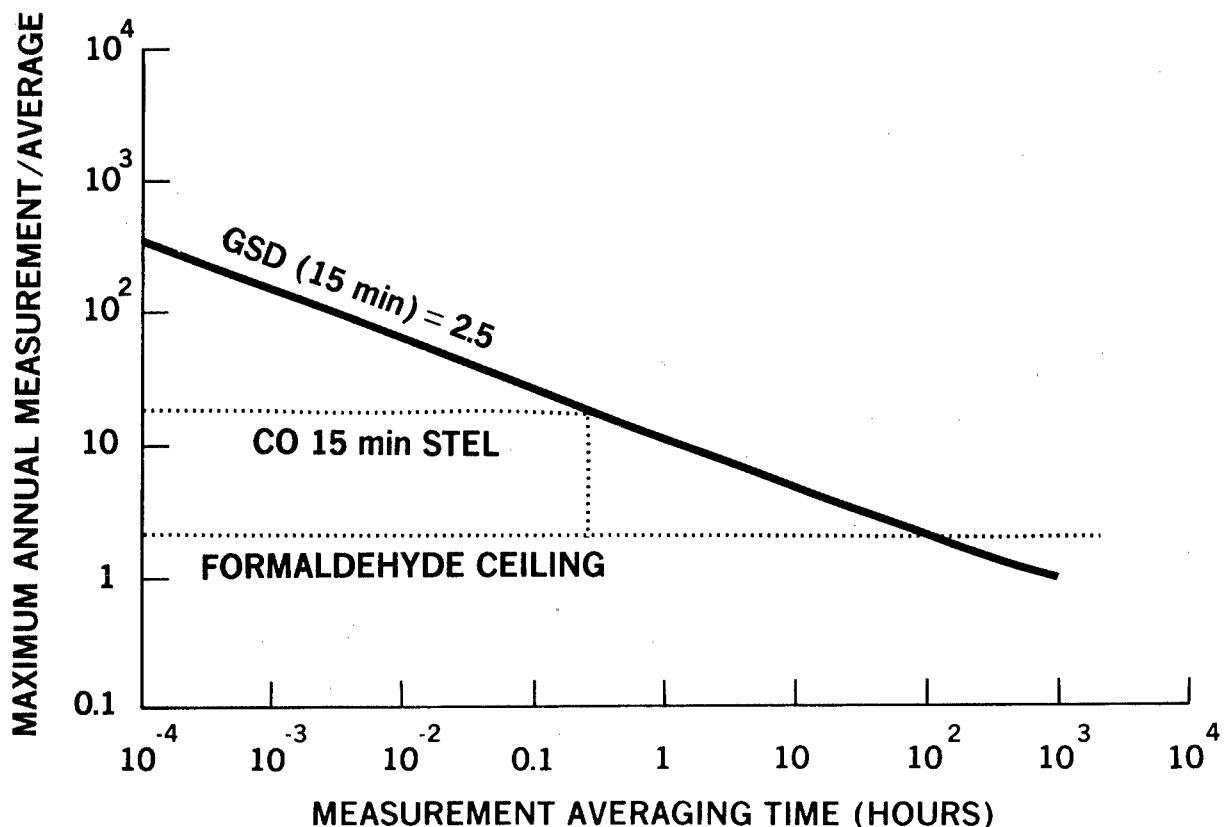


Figure 6. The annual maximum measurement (normalized to the annual average exposure, \bar{x}) as a function of the measurement averaging time. The dotted lines show the permissible exposure limits for the formaldehyde and carbon monoxide examples, drawn to scale. For averaging times longer than 15 minutes, both examples assumed that \bar{x} was one-half the PEL.

The practicing industrial hygienist, when faced with a situation like this formaldehyde example, has to decide if the workplace is acceptable. Using the traditional 30 minute sampling period with a midget bubbler and the criteria portrayed in Figure 2, the conclusion would be that the exposures are acceptable. If a shorter observation period were used, so that the occasional short excursion above 2 ppm were noted, an industrial hygienist would likely base his decision on the information in the ACGIH TLV Documentation (ACGIH, 1980). If he believes irritation is the reason for the ceiling designation he might still accept the workplace. If, however, he believes that short transient exposures above 2 ppm can contribute to irreversible health effects, then any indication of such excursions would likely justify corrective action in the workplace.

FRACTION OF MEASUREMENTS EXCEEDING PEL

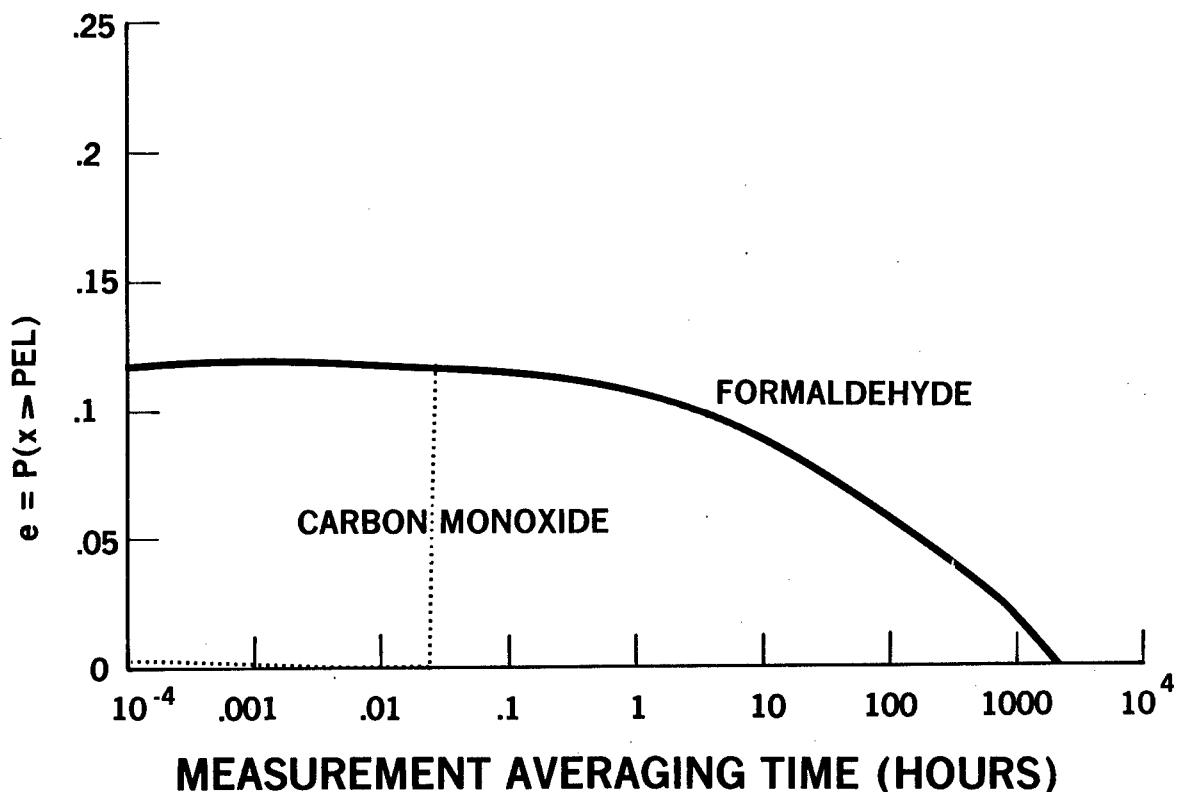


Figure 7. The fraction of all possible non-overlapping measurements exceeding the standard for the formaldehyde (solid line) and the carbon monoxide (dotted line) examples. In both examples $\bar{x} = (0.5) x$ (PEL at 8 hours). Because the PEL for CO is relaxed for periods less than 15 minutes, $\bar{x} = (1/16) x$ (PEL at 15 minutes).

The TLV with a C notation provides incomplete guidance as to proper disposition of the workplace. Only by applying judgments in toxicology to the measurements can an industrial hygienist decide whether to recommend expensive workplace modifications. But these judgments are the very ones that the professional environmental toxicology community declined to make when they established the ceiling standard without specifying an averaging time. The way out of the dilemma is to require an explicit averaging time for every short-term exposure limit. The ease of using such explicit standards is illustrated in the second example.

For the second example, consider carbon monoxide. The 8 hour standard is 50 ppm and the 15 minute standard is 400 ppm. Again to conform to assumptions of Figures 5 through 7, assume that the long-term average exposure is 25 ppm and that the environmental variability is such that the GSD of possible sets of measurements is accurately portrayed in Figure 5: $GSD = 2.5$ for 15 minute measurements, and $GSD = 2.05$ for 8-hour measurements. The long-term average exposure equals half the 8-hour standard, and so the workplace plots in Figure 2 at the point where the $\bar{x} = 0.5$ contour crosses $GSD = 2.05$. This is well within the probably acceptable region of Figure 4. Similarly, the long-term average exposure equals one-sixteenth the 15-minute standard, and so the workplace plots in Figure 2 at the point where the $\bar{x} = 1/16$ contour (very close to the GSD axis) crosses $GSD = 2.5$. This point, too, is well within the presumably acceptable region defined for 8-hour exposures. It is so close to the GSD axis that the workplace appears to provide an environment free from the recognized hazards of carbon monoxide.

Figure 6 shows the characteristic maximum annual measurement and the applicable PEL as a function of averaging time for both examples. The characteristic maximum annual measurement is that level which we expect the largest measurement in a complete set of nonoverlapping measurements to exceed (Gumbel, 1958). The discontinuity in the carbon monoxide curve is due to the discontinuity in the exposure standard at 15 minutes. Rather than relaxing the permissible exposure standard by interpolating for averaging times between 15 minutes and 8 hours, it is conservatively assumed that the 8 hour standard applies to all averaging times longer than 15 minutes. Because of the detailed toxicodynamic information available for carbon monoxide, this conservative assumption is really unnecessary, but it illustrates another case where the practicing industrial hygienist is called upon to make a judgment which the professional environmental toxicology community has not yet incorporated into practical exposure standards (Brief, 1975). Figure 6 illustrates the fact that an occasional exposure above the standard is expected, even in presumably acceptable workplaces. Once a year a 15 minute measurement is expected to exceed the standard slightly while an 8 hour measurement is expected to exceed twice the standard for CO. The formaldehyde standard is expected to be exceeded by a factor of 16 for 15 minute measurements and by a factor of 100 for 0.0001 hour measurements.

Figure 7 shows the fraction of measurements, e , which exceed the PEL from Figure 6 for both examples. Two noteworthy features of occupational exposures are illustrated. First, the formaldehyde curve illustrates the distinction between the maximum annual measurement and the fraction of measurements exceeding the standard. The maximum measurement increases with the number of non-overlapping measurements. The fraction of measurements exceeding the standard remains relatively stable with fewer than 13% of measurements exceeding the standard, even for very short observation

periods (and with $\bar{x} = 0.5$). Second, the carbon monoxide shows that when the short-term exposure limit is less restrictive than the long-term limit, the fraction of exposures exceeding the standard can be small for short measurements. This observation suggests that a simple tabulation of the fraction of measurements exceeding the PEL might be a useful regulatory mechanism, to replace the current concept of using the largest measurement.

To this point in the discussion, decisions about workplace quality have been based upon the long-term average exposure and on the variability reflected by the GSD of a set of measurements. Figure 5 clearly shows that the measurement GSD is a strong function of the measurement averaging time. Logically, it should also be a function of the intrinsic variability of the workplace. Let the GSD of 15-minute measurements define the intrinsic variability of workplaces. Figure 8 shows measurement GSD values as a function of workplace variability and averaging time. Figure 8 is based upon a model of several years of ambient air measurements (Larsen, 1971) and the assumption that except for scale, there is little difference between the macrometeorology of ambient air monitoring and the meso- or micro-meteorology of most industrial workplaces. This assumption of the equivalence between the statistics of occupational exposure measurements and those of ambient air quality measurements needs to be checked experimentally, and is offered here as an open research question for anyone able to address it.

MEASUREMENT GSD vs AVERAGING TIME

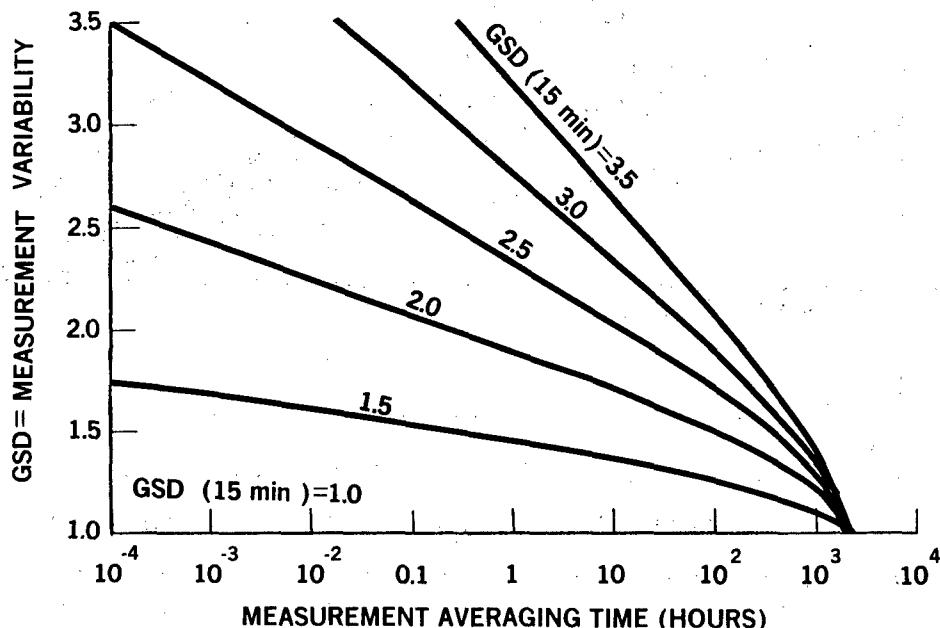


Figure 8. An illustration that measurement variability is a function both of averaging time (as in Figure 5) and of the intrinsic variability of a workplace, here defined to equal GSD (15 min).

Figure 9 is derived from Figure 8 using previously published concepts (Larsen, 1977; Gumbel, 1958). These data are most easily understood if the two extreme cases are clearly portrayed. The bottom curve represents a typical toxic exposure study where the concentration remains constant with no variation. Indeed, $GSD(15\text{ min}) = GSD(\text{any averaging time}) = 1$. In contrast, the top curve represents a worst case maintenance worker who had an exposure equal to $(2000) \times (\text{PEL})$ during one 15 minute period and no exposure for the rest of the year. Although the GSD is not defined for this case, the annual maximum measurement is easily computed in multiples of the PEL : $(2000/4) = 500$ for 1-hour measurements; $(2000/32) = 62.5$ for 8-hour measurements; and $(2000/160) = 12.5$ for 40-hour measurements. Workplaces with intermediate amounts of variability are plotted between these two extreme cases and labeled with the GSD representing the variability of 15 minute measurements.

MAXIMUM ANNUAL MEASUREMENT

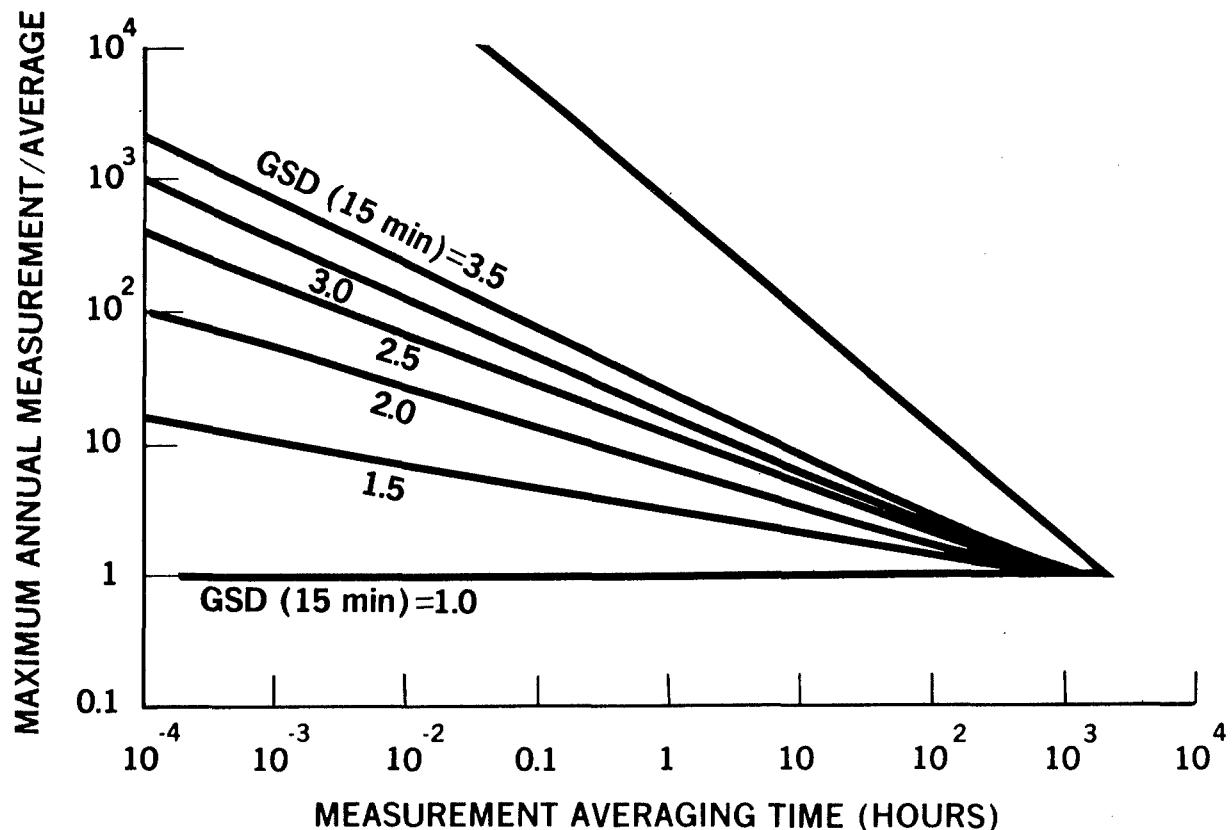


Figure 9. The annual maximum measurement is a function of both averaging time and the intrinsic variability of the workplace, $GSD(15\text{ min})$.

REAL MEASUREMENTS

Real measurements have a broader distribution than the ideal measurements used to illustrate the need to specify an averaging time. Statistical estimation procedures are used to compensate for the random error introduced by air sampling and subsequent chemical analysis. One of the most widely publicized of these is the action level concept (Leidel, 1975). It was introduced to permit an efficient decision in clean workplaces. If one representative sample is less than the statistically derived action level, then the workplace may be assumed to be acceptable with 95% confidence. The null hypothesis for the NIOSH Action Level is that the probability 5% of exposures exceeding the standards is less than 5%.

A variation of this concept, called the AEL Action Level, uses the null hypothesis that the probability that the long-term average exposure exceeds 95% of the standard is less than 5%. The decision regions for this test statistic are plotted in Figure 10 (Rock, 1981c and 1982a). Only those workplaces in the horizontally shaded area have a probability greater than 50% of being accepted by the AEL AL test. Figure 11 shows the maximum probable exposures for those workplaces likely to pass each of four action level tests. Two of these tests are rigorously derived test statistics: NIOSH AL and AEL AL. Two are pragmatic tests which are easy to apply but fail to take into account the measurement variability; legal AL and OSHA AL. The pragmatic tests do not limit the maximum probable exposure.

DECISION CONTOURS: AEL ACTION LEVEL

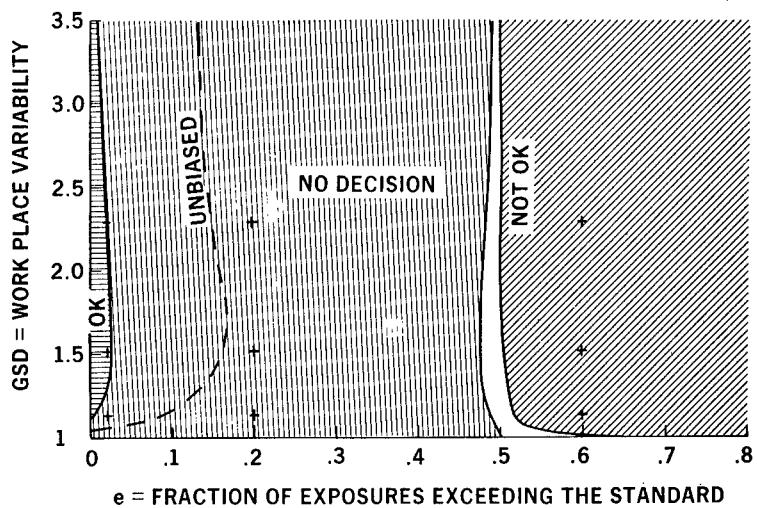


Figure 10. Decision Regions showing which workplaces are likely to pass or fail the statistically derived average exposure limit (AEL) action level criteria based upon 95% confidence that the long-term average exposure is less than 95% of the 8-hour PEL (Rock, 1981).

MAXIMUM ANNUAL 8-HOUR MEASUREMENT

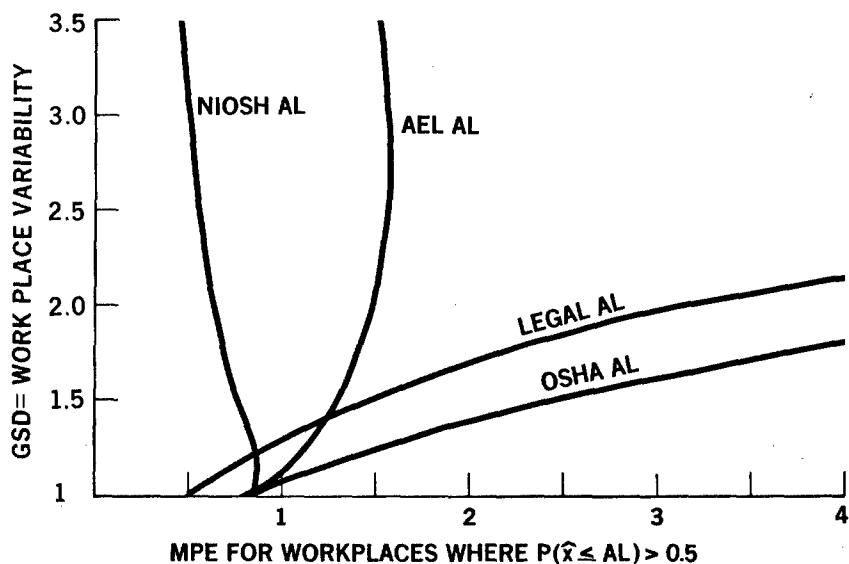


Figure 11. The maximum probable annual daily exposure is plotted as a function of the workplace variability, GSD (8-hour), for the dirtiest workplace likely to pass each of four action level decision criteria. Although only the AEL action level criteria are described in this paper, the others have been previously described (Rock, 1981c). Note that statistically based criteria such as the NIOSH action level and the AEL of Figure 11 which are designed to control the annual average exposure, actually limit the maximum daily exposure when implemented. Pragmatic and non-statistical approaches such as the OSHA compliance criteria or the Legal Action Level which ignore day-to-day variability (by assuming GSD (8-hour) = 1.0) do not create this synthetic safety factor.

However, both statistically derived AL tests do limit the maximum probable exposure. This is not surprising for the PEL AL since it was designed to limit the maximum 8-hour measurement. The AEL AL test is particularly noteworthy, since it was designed to limit only the 2000-hour measurement. It also effectively limits the 8-hour measurement to less than 160% of the 8-hour PEL, no matter what the variability of the workplace. If the AEL were smaller than 95% of the 8-hour PEL, the maximum probable 8-hour exposure would be limited to even smaller values. This is the first example of the consequences of dual exposure limits. When implemented, one limit usually dominates, unless both the long-term and the short-term standards are designed to be complementary in an acceptable workplace.

CRITERIA FOR ESTABLISHING DUAL EXPOSURE LIMITS

The two examples given illustrate that a practicing industrial hygienist has difficulty assessing the meaning of exposures which differ in duration or in concentration from those specified in the PEL. Suggestions for dealing with this problem have been published. Brief and Jones (1976) correctly pointed out that the combination of a short-term and a long-term standard may represent a rather complete description of the distribution of measurements in a workplace if a lognormal distribution can be assumed. Rapaport et al. (1981) have developed a rigorous test statistic to allow measurements to be compared with a criterion limiting distribution which is assumed to represent an acceptable workplace.

The nagging question remaining is how to define the limiting distribution. This question is particularly troublesome in light of the earlier discussions which clearly show that the distribution of measurements is a function of the measurement averaging time. Thus it seems prudent to examine the averaging process before establishing detailed criteria for the occupational exposure standards setting process.

THE ROLE OF AVERAGING IN OCCUPATIONAL HEALTH

The effects of averaging in occupational health have been treated occasionally in the professional literature (Saltzman, 1970; Roach, 1966 and 1977). Suffice it to say here that just as industrial hygiene sampling is an averaging process, so also is the toxicodynamic response of any biological organism. Figure 12 compares the Fourier Transform of the industrial hygiene sampling process (moving-time average) with that of a typical 1st order system characterized by a single dominant time constant (exponential average) (Rock, 1981b). With a simple horizontal shift of the moving-time average curve, it can be made into a good approximation of the exponential average time constant. Indeed, the noise bandwidths of the two processes are nearly equal if the moving-average time constant is set equal to 67% of the exponential time constant. In other terms, if the average time for the PEL is set equal to the biological half-time of the toxicodynamic process, then the variability of industrial hygiene measurements will approximate the variability of the toxic substance body burden due to environmental exposures (Rock, 1982b).

Figure 12 contains the information needed to select the optimum measurement parameters for specified purposes. Comparing the usual systems engineering assumptions with industrial hygiene sampling criteria yields useful new insights which are summarized in Table 1.

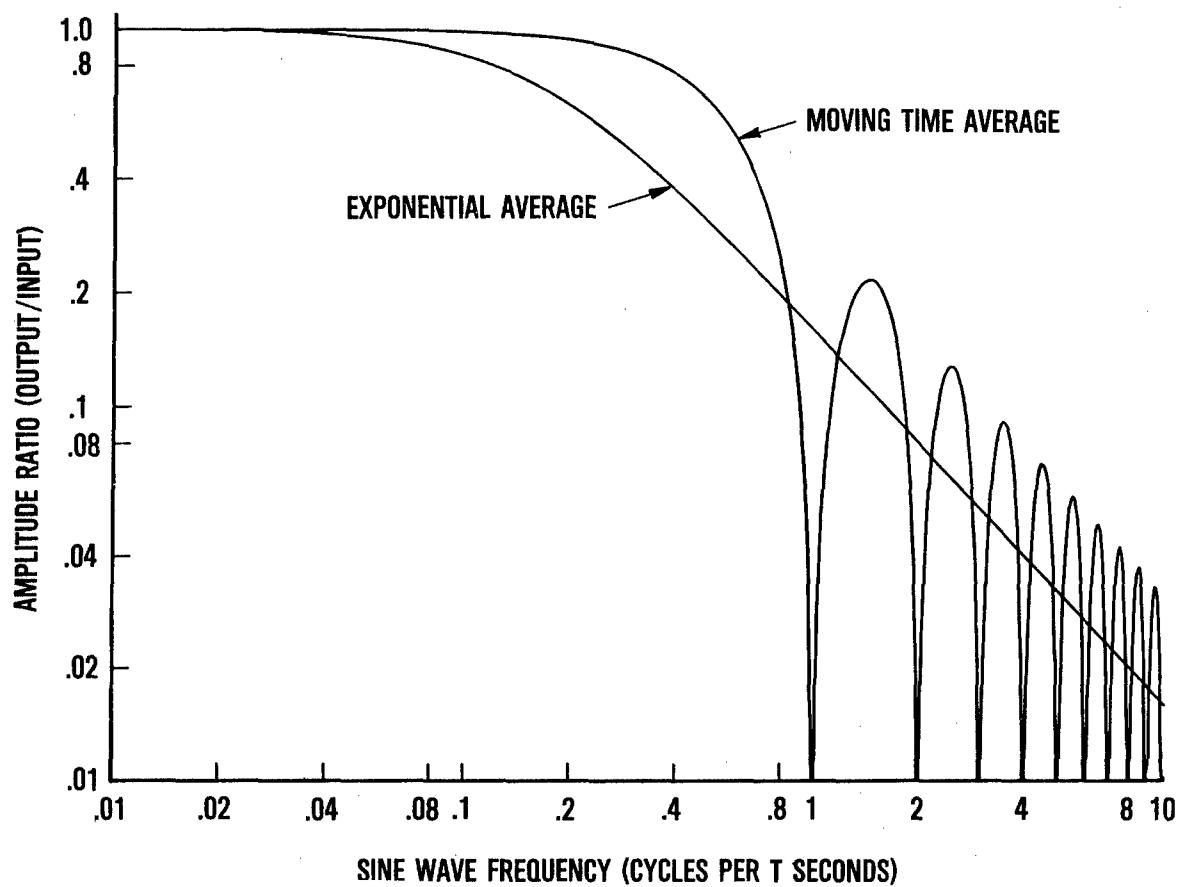


Figure 12. Frequency response curves for both exponential averaging (toxicodynamic or direct-reading instrument response) and for moving-time averaging (typical industrial hygiene samples). The frequency axis is normalized to the averaging time constant, T . For the moving average, T is equal to the duration of a sample. For the exponential average, T is equal to the exponential time constant (Rock, 1981b).

TABLE 1. CHOOSING OPTIMUM INSTRUMENT PARAMETERS

MEASUREMENT OBJECTIVE	INSTRUMENT PARAMETERS		
	T_{exp}	T_{ma}	W_s
SIGNAL STATISTICS ($T_a = 1\% \text{ POINT ON } R(t)$) ($W_x = 1\% \text{ POINT ON } S(w)$)	$0.15/W_x$ $T_a/3$	$0.4 W_x$ $0.8 T_a$	$2W_x$
TWA, STEL, C (T_s FROM STANDARD)	$1.5 T_s$	T_s	$2/T_s$
BODY BURDEN (T_h - HALF LIFE)	$1.4 T_h$	T_h	$2/T_h$

* PICK THE SMALLER VALUE.

T_e = INSTRUMENT TIME CONSTANT. T_{ma} = MOVING AVERAGE TIME CONSTANT.

W_s = DIGITIZING RATE

PARAMETERS FOR PEAK DETECTION

The usual communications engineering assumption is that 3 dB is the maximum tolerable loss during signal processing (output/input = 0.707). Note that this is nearly the same as the NIOSH requirement of no more than $\pm 25\%$ sampling and analytical error for concentrations near the permissible exposure limit. Using these criteria, we find from Figure 12 that when the exponential time constant of a direct-reading instrument exceeds 16% of the period of the input signal, the output signal falls to less than 70.7% of the input signal amplitude (measured on a peak-to-peak basis). Likewise, when the moving-average time constant exceeds 44% of the period of the input signal, then peak-to-peak variation of the output signal falls to less than 70.7% of that of the input signal.

Thus, if we want to estimate the true peak exposures in the workplace, we should choose direct-reading instruments with exponential time constants shorter than 15% of the period of a periodic input signal. If we must use a grab sampling technique, we

should choose a method which has adequate sensitivity with a sampling time of no more than 40% of the period of a periodic input signal. If the input signal is random, then the significant peak width may be estimated by T_a , the 1% width of the autocorrelation function. In this case, the exponential time constant should be shorter than $T_a/3$ and moving-average time constant should be less than 0.8 T_a to preserve the peak values of random signals (Rock, 1982b). Only by following these restrictions can we hope to create an accurate impression of the true peak exposures in the workplace. Note that if the time constant is longer than specified in line one of Table 1, peak exposures will be underestimated by more than the 25% error tolerance that NIOSH recommends for air monitoring.

PARAMETERS FOR AVERAGE MEASUREMENT

On the other hand, if we are interested in measuring the average exposure by averaging out the peak exposures, then we need to select a longer time constant. The usual systems engineering assumption is that unwanted peaks should be attenuated by at least 20 dB (output/input = 0.1). From Figure 10, it is easily seen that the time constant for a moving average operator should be at least 3.0 times as long as the period of a periodic input signal, and that the exponential time constant should be at least 1.5 times longer than the period of the input signal to create good averaging.

If nonperiodic signals are involved, we should select large time constants to filter out the variance of the random process. It is not always easy to say how large, but some guidance is possible. If averaging is the objective, the time constant should never be less than three times the maximum time constant specified to insure good peak detection. Thus, $T_e > T_a$ and $T_{ma} > 2.4 T_a$.

PARAMETERS TO MEET REGULATORY STANDARDS

However, if the purpose of the measurement is for comparison with a standard which specifies a sampling time T_s , then the appropriate instrument time constants are $T_e = 1.5 T_s$ and $T_{ma} = T_s$. This insures that the variability of measurements is comparable, independent of whether conventional integrated sampling procedures or direct reading instruments are used. If the measurement time constant is shorter (longer) than specified in line two of Table 1, then the variance and consequently the peaks of the measurement will be larger (smaller) than intended by the standard.

PARAMETERS FOR SIMULATING TOXICODYNAMIC SYSTEMS

Finally, industrial hygiene sampling may require other considerations. If the biological half-life Th is known for the contaminant of interest, then it is appropriate to set $Te = 1.4 Th$ so that the instrument output approximates the time-history of biological body-burden. If moving-time averaging is used, $Tma = Th$ will provide measurements which faithfully approximate the variability of the body burden. If the measurement time constant is shorter (longer) than specified in line 3 of Table 1, then the variance and the peaks of the measurements will be larger (smaller) than the variance and peaks of the body burden of exposed employees.

RULES FOR SETTING PERMISSIBLE EXPOSURE LIMITS

Although it has been a rapid overview rather than a detailed development, the necessary background for rational standard setting is in hand. The first prerequisite is knowledge of the toxicodynamic time constant for the body burden. If there are several toxic mechanisms, then each one of them should be listed. Fortunately, the kinetics of even complex metabolic pathways are normally dominated by a single rate limiting step. Two common examples are flow limited and membrane limited steps. The next prerequisite is a maximum tolerable body burden for each toxic mechanism and the associated airborne threshold concentration which produces that steady state body burden. If the environmental toxicology community can generate these kinds of data, the following algorithm may be a useful standard-setting tool.

Table 2 shows that preliminary standards are created by associating each biological time constant (expressed as a half-life) with its steady state threshold concentration. These pairs are then normalized to the lowest threshold concentration in the set of thresholds and plotted on Figure 9. Pick the most variable workplace lying below all of these points as indicative of the maximum variability which can be tolerated without exceeding the known body burden limits. Read the values for the 15-minute PEL and the 8-hour PEL from that contour.

These preliminary standards would represent exposure standards with no explicit intrinsic safety factor. There are many ways that safety factors could be incorporated into this standard setting formalism, but it seems premature to discuss those until the general proposal has received adequate peer review. There are some subtle intrinsic safety factors already present in this formalism, and they are worth mentioning.

TABLE 2. ALGORITHM FOR CREATING PERMISSIBLE EXPOSURE LIMITS

1. *List all toxic mechanisms.*
2. *List biological half-times for each mechanism from 1.*
3. *List tolerable body burden for each toxic material or metabolite from 1 and 2. Call these threshold body burdens.*
4. *List the steady airborne contaminant concentration needed to produce the steady threshold body burden from 3.*
5. *Normalize all concentrations to the smallest concentration listed in 4.*
6. *On Figure 9 plot all pairs of half-time from 2 against twice the normalized concentration from 5. (This accounts for the fact that only half of the body burden can be reached in one half-time.)*
7. *Select the steepest contour lying below all plotted points and read off normalized concentrations for 15-minute and 8-hour PELs.*
8. *Multiply by normalization factor from 5 to create PELs that prevent biologically dangerous peak exposures.*

IMPLICIT SAFETY FACTORS

One of the assumptions of the model which is not met in practice is that all measurements are uncorrelated and independent of all other measurements. Although this may be true to some extent in the ambient air data Larsen used, it can be less so in workplace measurements (Roach, 1981). As a consequence, the maximum probable exposures do not increase indefinitely with shorter and shorter sampling times as is implied in Figure 9. Secondly, there is a maximum possible concentration for many compounds found in the workplace environment. This maximum exists due to the physical properties of the material itself or of its condition of use. For both of these reasons, the model overestimates the size of the shorter transient peak exposures. Since the proposed standard-setting procedure establishes longer-term standards at a level designed to prevent dangerous short peaks, the long-term standards will tend to be set lower than necessary.

A second assumption of the model is that the biological response to a toxic compound can be represented by a linear system. This is clearly not the case in general, and in particular it is well known that not only are metabolic pathways characterized by

rate-limiting steps but also that they are characterized by saturable steps (Andersen, 1981). This phenomenon is not unknown to systems analysis. When the saturable step precedes the creation of the biologically dangerous situation, then it represents a safety factor, since large transient exposures create less danger than predicted. On the other hand, if the saturable step precedes the detoxification mechanism, then the danger is increased since the body burden will be larger than was assumed in setting the standard. This problem, so long as it is recognized, can be accounted for in the standard setting process. It is mentioned here because this author firmly believes that the proper place for the decision lies in the standard setting environment; it should not be deferred to the time when the standard is applied in the workplace.

Another conservative error occurs when the standards are actually used. Most statistical sampling strategies assume that the distribution of exposures in the workplace is stationary over time. Then, on the basis of a few representative measurements, the workplace variability is estimated in order to discover whether or not occasional extreme exposures are in violation of the PEL. It is well known that most workplaces exhibit nonrandom trends. The seasonal trend is one of the most universal of these trends. The presence of any nonrandom trend causes statistical estimation procedures to overestimate the variance of the measurements (Horowitz, 1979). Thus the peak exposures are overestimated and a *de facto* safety factor is often created at the time the standard is applied.

Finally, consider the effect of nonideal sampling/analytical procedures which introduce some nonenvironmental variability into the data. This also increases the variance of the measurements, causing peak exposures to be overestimated by simple decision rules. Statisticians are very adept at correcting for this variance with sophisticated test statistics such as action levels (Leidel, 1975), one-sided tolerance tests (Tuggle, 1982), and limiting distributions (Rappaport, 1981). However, in order to avoid well-known type I and type II errors, the test statistics are usually constrained by a requirement for 95% confidence in the decision. In practice, this makes it even less likely that a marginally acceptable workplace will be declared acceptable.

CONCLUSION

This paper, falling as it does between professional specialties, should be viewed as the first draft of a proposed formalism for the creation of permissible exposure limits. There may be errors of omission or errors of commission. If so, they are unintentional and the author will gratefully accept all constructive criticism.

The proposal itself is based upon easily stated principles. Workplace measurements preserve certain features of the workplace environment while irretrievably discarding other features. Standards should be set so that the measurements preserve those features of the workplace environment which are most representative of the biological risk. In order to do so, those standards should be based upon toxicodynamic information. Knowledge of the metabolic pathway sufficiently detailed to define both the toxicodynamic time constant and the steady state concentration which leads to a threshold body burden is sufficient to create an exposure standard with an unconventional sampling time. Knowledge of the workplace sampling process is sufficient to convert this unconventional specification into two conventional permissible exposure limits, a 15-minute standard and an 8-hour standard. In some cases additional standards might be appropriate (1 minute, 5 minutes, 1 year). The slope of the inverse relationship between an exposure standard and its specified averaging times limits the permissible workplace variability in such a fashion that biologically dangerous peak exposures are prevented.

APPENDIX

MATHEMATICAL BASIS FOR ILLUSTRATIONS

The normalized exposure variable, x , is computed by dividing an exposure by the applicable permissible exposure limit.

$$x = \text{exposure/PEL} \quad (1)$$

The set of 250 possible nonoverlapping daily exposures occurring during a typical working year (50 weeks at 5 days/week) is approximately lognormally distributed. The probability density function illustrated in Figure 1 is given by equation 2.

$$f(x) = [1/\{x\sqrt{2\pi \ln(GSD)}\}] \exp(-0.5 [\{\ln(x) - \ln(GM)\}/\ln(GSD)]^2) \quad (2)$$

The probability density function is completely specified by the geometric mean, GM and the geometric standard deviation, GSD. The average exposure, \bar{x} , is plotted in Figure 2 and given by equation 3.

$$\bar{x} = \exp[\ln(GM) + 0.5 \{\ln(GSD)\}^2] \quad (3)$$

Two parameters are of industrial hygiene interest: e = the fraction of exposures exceeding the standard and MPE = the maximum probable exposure or that daily exposure which is expected to be exceeded only one day per 250 day working year.

$$e = P(x > 1.0) \quad (4)$$

$$P(x > MPE) = 1/250 \quad (5)$$

To calculate numerical values for e and MPE, it is necessary to introduce the standard normal Z-variate. It represents a one-to-one mapping between a = the area in the lower tail of the standard normal distribution, and the associated value $Z(a)$, given in units of the standard deviation.

$$a = [1/\sqrt{2\pi}] \int_{-\infty}^{Z(a)} \exp(-u^2/2) du \quad (6)$$

The variables, a and u , in equation 6 are dummy variables which take on defined values in order to calculate values for e and MPE used in Figures 2 through 4.

$$Z(e) = \ln(GM)/\ln(GSD) \quad (7)$$

$$\ln(MPE) = \ln(GM) + Z(249/250) \ln(GSD) \quad (8)$$

Industrial hygienists deal only in measurements, not in exposures. It is reasonable to assume that the set of all possible nonoverlapping measurements collected during a 2000-hour working year (50 weeks at 40 hours/week) is lognormally distributed. If the measurement averaging time is t' hours, then there are $2000/t'$ nonoverlapping measurements during a working year. Under conditions that seem reasonable for industrial hygiene measurements, it has been shown that the geometric standard deviation of a set of measurements taken with an arbitrary averaging time of t' hours can be computed from the observed variability of quarter hour measurements.

$$\ln(GSD_{t'}) = [\ln(GSD_{0.25})] [\sqrt{\ln(2000/0.25)/\ln(2000/t')}}] \quad (9)$$

The arithmetic average value of all possible nonoverlapping measurements is independent of the measurement averaging time, t' , but the median of the measurements (which equals the geometric mean

of a lognormal distribution) is a function of the averaging time, as can be seen by rearranging equation 3 in light of equation 9.

$$\ln(GM_{t'}) = \ln(\bar{x}) - [0.5] [\ln(GSD_{t'})]^2 \quad (10)$$

Equation 8 is easily generalized to permit estimation of the maximum probable measurement as a function of measurement averaging time.

$$\ln(MPM_{t'}) = \ln(GM_{t'}) + [Z(1-t'/2000)] [\ln(GSD_{t'})] \quad (11)$$

By subtracting $\ln(\bar{x})$ from both sides of equation 11 and combining with equation 10, one can derive a convenient expression for plotting the ratio of the maximum probable measurement expected during a year to the more easily estimated average of all possible measurements.

$$\ln(MPM_{t'}/\bar{x}) = [Z(1-t'/2000)] [\ln(GSD_{t'})] - [0.5] [\ln(GSD_{t'})]^2 \quad (12)$$

A systems engineering approach is useful when considering the relationship between industrial hygiene measurements, modeled as a moving-time averaging process in equation 13, and physiological response, modeled as an exponential averaging process in equation 14. Both the time domain and the frequency domain formulas are given. T is the averaging time constant, u is a dummy variable of integration, t is the time in seconds, w is the frequency in radians/second, and j is the square root of minus one.

For a moving-time averaging process:

$$\bar{x}(t) = [1/T] \int_{t-T}^t x(u) du; \quad \bar{X}(jw) = [1/\{1 + jwT\}] \quad (13)$$

For an exponential averaging process:

$$\bar{x}(t) = [1/T] \int_{-\infty}^t x(u) e^{[(u-t)/T]} du; \quad \bar{X}(jw) = [1 - e^{-jwT}] / [jwT] \quad (14)$$

To summarize, then, Figure 1 is based on equation 2; Figure 2 is based on equations 3, 6, and 7; Figure 3 is based on equations 3, 6, 7, and 8; Figure 4 is based on Figures 2 and 3; Figures 5 and 8 are based on equation 9; Figures 6 and 9 are based on equation

12; Figure 7 is based on equations 6, 7, and 10; and Figure 12 is based on equations 13 and 14. The derivation of equations for Figures 10 and 11 is beyond the scope of this paper, but has been published elsewhere (Rock, 1981c and 1982a).

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**EVALUATING HEALTH HAZARDS ASSOCIATED WITH
AIRCRAFT FUEL CELL MAINTENANCE**

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INTRODUCTION

The purpose of this paper is to demonstrate problems commonly encountered when toxicologic standards are applied to everyday worker exposures. The primary function of the industrial hygienist is to evaluate the workplace to insure a healthful environment for the worker. In general, this evaluation is accomplished by taking samples representative of the worker's environment, analyzing for contaminants of interest, and comparing the measured levels with appropriate standards. Control measures are instituted if contaminant levels are above the standard or some preset fraction of the standard (NIOSH action level concept). Although the evaluation procedure may seem simple as described, it is a nontrivial task as demonstrated using the example of a recent evaluation of worker exposures during aircraft fuel cell maintenance.

Aircraft fuel cell maintenance is concerned with the detection and repair of leaks in aircraft fuel cells and integral tanks. The major operations involved in the repair of leaks are described below⁽¹⁾ and Table 1 shows the exposure agents generally associated with each operation.

TABLE 1. FUEL CELL MAINTENANCE OPERATIONS

Defuel/Purge - JP-4 and JP-5
Depuddle - JP-5 and JP-4 light ends
Clean - 4-part cleaner or MEK
Deseal/Seal - sealants
Hot Leaks - JP-4, 4-part cleaner, sealants
Bladder Removal - JP-4 and JP-5
Foam Removal - JP-5 and JP-4 light ends

(1) T.O. 1-1-3 (1980), Technical Manual, Inspection and Repair of Aircraft Integral Tanks and Fuel Cells, USAF.

- a. Defuel/Purge: The purpose of the defuel and purge operation is to lower the combustible vapor content in the tank or cell to the fire-safe level of 20% of the lower explosive level (LEL). The aircraft fuel is drained from the tanks and cells into fuel trucks and the residual is drained into fuel carts. The aircraft tanks are then refueled with the purging fluid (JP-5 or a similar light oil with a low vapor pressure) and the purging fluid is transferred between tanks to absorb the remaining fuel. The purging fluid is then drained from the aircraft and air purging is accomplished by blowing air through the tanks until the concentration is lowered to less than 20 %LEL.
- b. Depuddle: The workers enter the tank after the combustible vapor level has been lowered to less than the 20 %LEL entry level. Initially a vacuum cleaner is used to remove residual purge fluid, followed by hand mopping.
- c. Deseal/Cleaning: The area of the leak is desealed using a scraper and the area cleaned using a four-part cleaner or methyl ethyl ketone (MEK) prior to sealing.
- d. Sealing: Two sealants are applied to the leak area. The first sealant is a brush-on liquid which contains volatile components such as MEK and toluene. This is followed by the second less volatile sealant which is pneumatically ejected from a tube applicator.
- e. "Hot" Leaks: At operational bases the aircraft are often worked "hot" without the use of purge fluid. Aircraft are also worked "hot" at the depot if leaks are found after repairs are completed. During hot operations the fuel is pumped from the leaking tank into adjacent tanks; the leaking tank is opened, and air is blown in to reduce the concentration level to below 20 %LEL. Personnel then enter the tank and repair the leak using the procedures discussed above.
- f. Bladder Removal: Certain aircraft contain large rubber fuel bladders that are removed for repair. The aircraft is usually (but not always) fluid purged, followed by air purging, and then tank entrance.
- g. Foam Removal: Certain aircraft contain fire suppressant foam in the fuel tanks which must be removed prior to tank repair. In general the tanks are fluid purged and then air purged to below the 20 %LEL entrance requirement prior to foam removal. Again, at an operational base, the tank may be air purged without the use of the purge fluid.

The evaluation of the worker exposures during the various fuel cell maintenance operations is complicated by many factors:

(1) USAF personnel do not do the same job every day; one day they may be involved with defueling only, while the next they may be cleaning and sealing or replacing a broken pump in the tank;

(2) Exposures during the same operation will vary both within day and between days;

(3) Although inhalation is the primary route of entry of these agents, skin absorption can also be a major contributor and ingestion due to poor hygiene practices cannot be ruled out;

(4) Vapor is the predominant physical form of the exposure, but the possible presence of aerosols also exists;

(5) Evaluations are further complicated by exposures to multi-component agents which can be classified as special blends such as the cleaners and sealants, or to distillation-cut agents such as the jet fuels and purging fluids.

The remainder of this paper will focus on the problems associated with evaluating exposures to distillation-cut jet fuels.

FUEL EXPOSURE CHARACTERISTICS

JP-4 liquid is described as a wide-cut, gasoline type fuel which generally contains C₄ through C₁₆ hydrocarbons. JP-5 liquid is characterized as a high flashpoint, kerosene type fuel which generally contains C₈ through C₁₆ hydrocarbons⁽²⁾. Other characteristics are shown in Table 2^(2,3). These simple definitions mask complex mixtures which can contain 270 or more different components, not all of which have the same toxicologic significance or the same physical characteristics. The difference in physical characteristics leads to vapor compositions (exposures) which are significantly different from the evaporating liquid.

- (2) MIL-T-5264K (1976), Military Specification, Turbine Fuel, Aviation, Grades JP-4 and JP-5, USAF.
- (3) Fire Hazard Properties of Flammable Liquids, Gases and Volatile Solids (1977), NFPA 325M - 1977. National Fire Protection Association, Boston, Massachusetts.

TABLE 2. FUEL CHARACTERISTICS

DEF:	JP-4 Wide-Cut Gasoline-Type	JP-5 High Flashpoint Kerosene-Type
Carbon Range	C ₄ - C ₁₆	C ₈ - C ₁₆
LEL	1.3	0.6
UEL	8.0	4.5
vp at 100 F°	105 mm Hg	-----
Flash Point	-10 to 30 F°	95 to 145 F°

The vapor composition is a function of the liquid composition, x_i , the pure component vapor pressure of each component, $P_{o,i}$, and the activity coefficient of each component in the liquid, γ_i . This relationship is shown in the following equation⁽⁴⁾:

$$y_i = \frac{\gamma_i x_i P_{o,i}}{P} \quad (1)$$

where y_i = vapor phase mole fraction

γ_i = the activity coefficient

x_i = liquid phase mole fraction

$P_{o,i}$ = pure component saturated vapor pressure, mm Hg

P = total pressure, mm Hg

The activity coefficient corrects for deviations from an ideal liquid as expressed by Raoult's law (Figure 1). The activity coefficient is a very strong function of liquid composition as shown in Figure 2 and is moderately affected by temperature and pressure. The pure component vapor pressure is independent of composition but is very strongly influenced by temperature as shown in Figure 3.

(4) Treybal, R. E. (1968), Mass Transfer Operations, McGraw-Hill Publishing Company, New York, pp. 281-309.

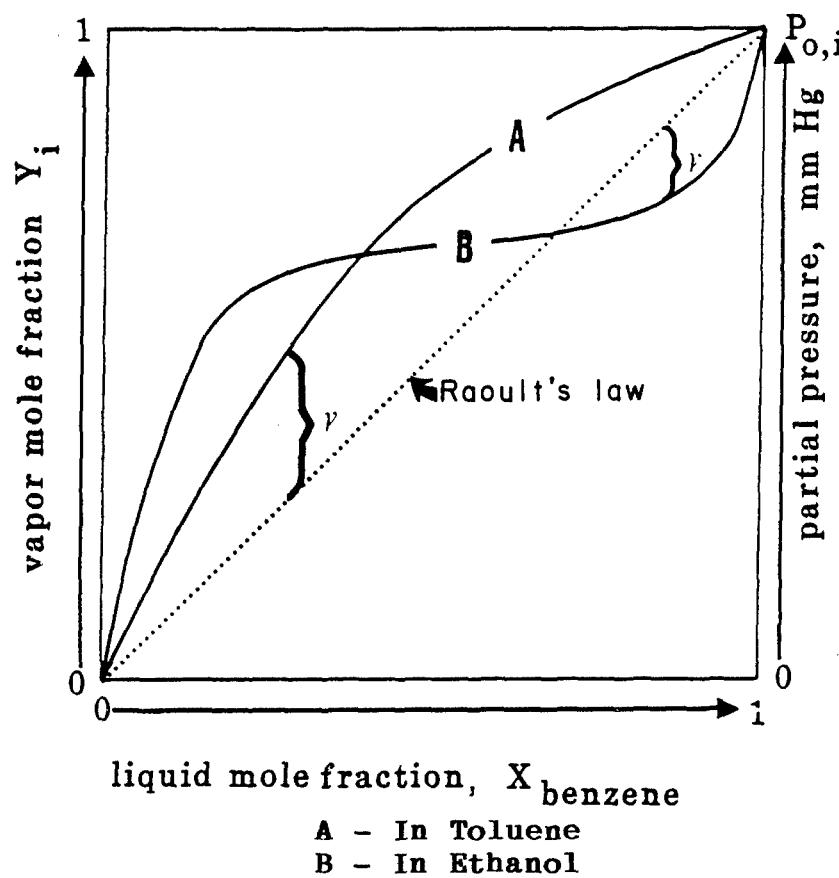


Figure 1. Vapor liquid equilibrium.

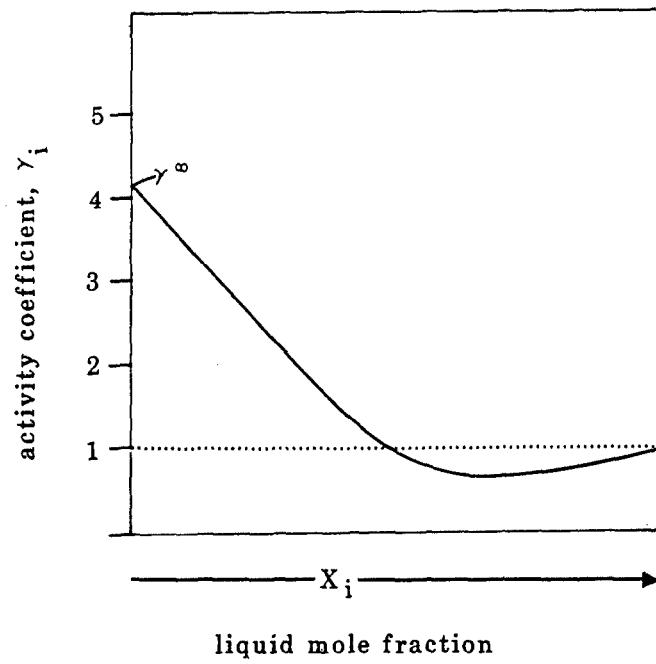
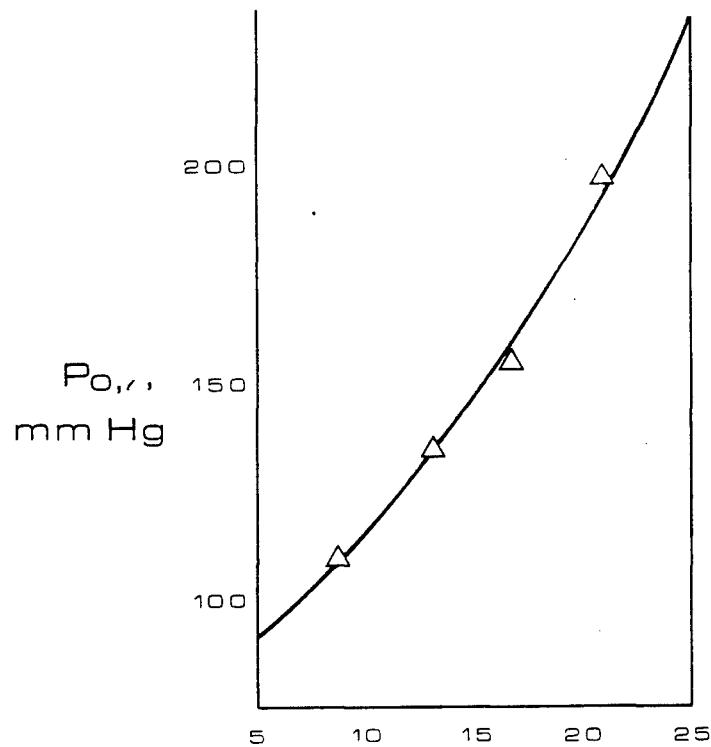


Figure 2. Composition dependence of activity coefficient.



$$\text{Log } P_{O,i} = A + \frac{B}{C + \text{Temp}}$$

Figure 3. Temperature dependence of pure component vapor pressure.

Relative volatility, α , is a measure of the difference in liquid and vapor composition⁽⁵⁾.

$$\alpha = \frac{Y_i x_j}{Y_j x_i} \quad (2)$$

(5) Smith, J. M. and H. C. VanNess (1975), Introduction to Chemical Engineering Thermodynamics, McGraw-Hill Publishing Company, New York.

When $\alpha = 1.0$, the liquid and vapor compositions are equal; the further from unity, the more different the liquid and vapor compositions.

Substituting for y_i and y_j from equation 1,

$$\alpha = \frac{y_i P_{o,i}}{y_j P_{o,i}} \quad (3)$$

Equation 3 shows that component vapor pressures and activity coefficients determine the composition of the vapor evaporating from a liquid. For a homologous series of hydrocarbons common to jet fuels, the activity coefficients will not vary greatly. However, the pure component vapor pressures will be significantly different. For example:

At 298 K° (25 C°, 77 F°)⁽⁶⁾

C₅, n-pentane, vapor pressure = 513 mm Hg

C₁₀, n-decane, vapor pressure = 1.3 mm Hg

The overall consequence of these interrelationships when applied to distillation-cut agents is a difference between vapor and liquid compositions. This difference can vary with time as the liquid composition changes due to evaporation of the more volatile components. Vapor and liquid composition differences will complicate any exposure evaluation where both skin absorption and vapor inhalation are possible routes of entry. Gas liquid chromatography chromatograms of bulk samples and headspace vapors of fuel and purging fluids are shown in Figures 4 and 5 and show the significant difference in vapor and liquid compositions predicted.

(6) Reid, R. C., J. M. Prausnitz, and T. K. Sherwood (1977), The properties of Gases and Liquids, 3rd ed, McGraw-Hill Publishing Company, New York.

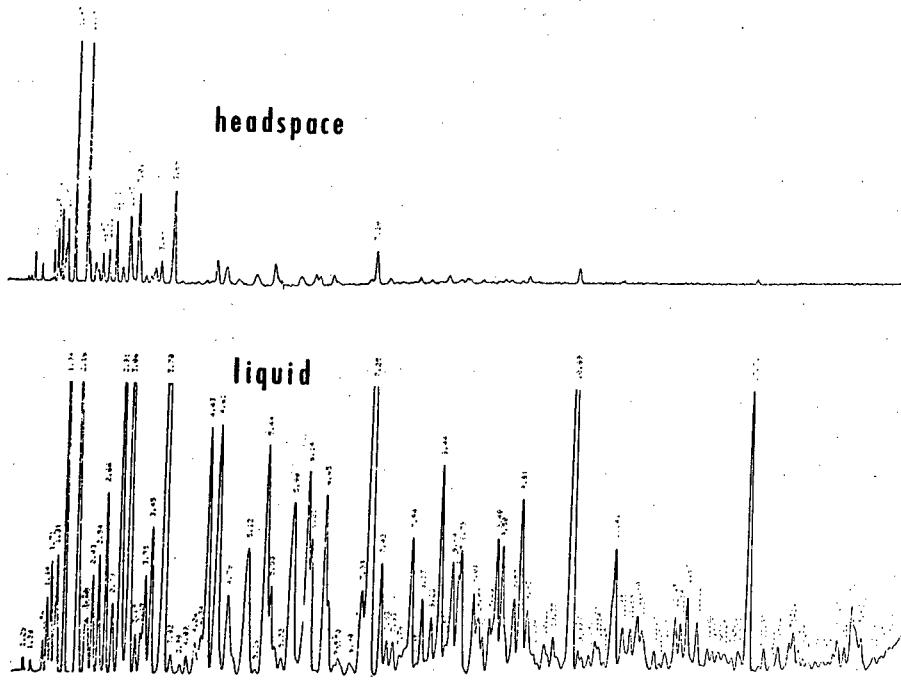


Figure 4. Bulk sample analyses, residual JP-4. Start times and charts are the same. NOTE: The purpose of this figure was to show that the headspace did not contain as many components as the liquid.

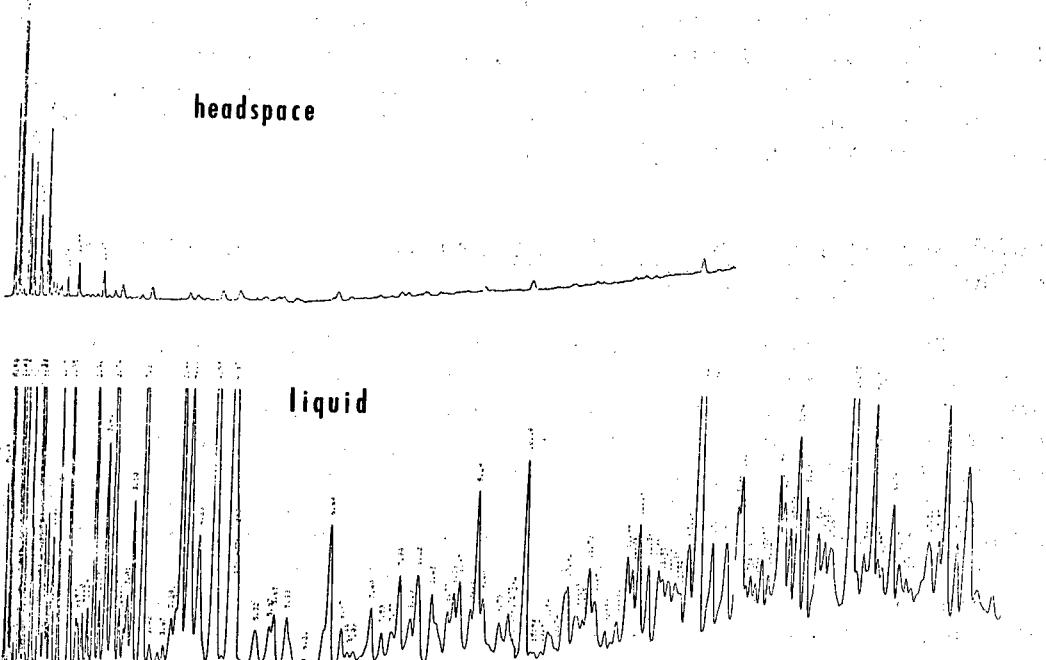


Figure 5. Bulk sample analyses, residual JP-5. Start times and charts are the same. NOTE: The purpose of this figure was to show that the headspace did not contain as many components as the liquid.

SAMPLING PROCEDURE

Personal and area samples were collected during the different fuel cell maintenance operations. Each sample consisted of two charcoal tubes in series, a DuPont organic vapor passive monitor and a 3M organic vapor passive monitor to provide an evaluation of the use of passive monitors in fuel cells.

Air was drawn through the charcoal tubes using a constant flow pump, so-called "active pumping". This method of sampling has the disadvantage of tubing connecting the pump to the charcoal tubes. We noted that the tubing was pinched closed several times when the worker entered or exited the fuel cell through the small access port. Additionally, because fuel cells can contain locally, potentially explosive atmospheres, we used special sampling pumps designed for safe use in Group A, B, C, and D combustible atmospheres.

Passive monitors use the principle of diffusion across a concentration gradient, "passive pumping", to transport the contaminants to the adsorption media. Although the passive monitors are easier to use because they do not require a sampling pump or tubing, they do require a knowledge of the sampling rate of the components of interest. This sampling rate is the rate of diffusion of each component applied to the geometry of the passive monitor. Diffusion coefficients have been experimentally determined for relatively few components and very little research has been done with mixtures. If experimental values are unavailable, the diffusion coefficient can be estimated, using one of several chemical engineering models. In the current study, we plan to calculate an average rate of diffusion based upon the average molecular weight of the vapor.

PROBLEMS ASSOCIATED WITH EVALUATION OF EXPOSURES TO COMPLEX HYDROCARBON MIXTURES

The common analytical procedure for hydrocarbons, including fuels, is gas-liquid chromatography (GLC) with a flame ionization detector (FID). The FID is generally used as the GLC detector for hydrocarbons due to its sensitivity and large linear range. However, the FID response is not identical for all hydrocarbons as shown in Table 3. The relative response factor for most hydrocarbons commonly found in jet fuels is from approximately 0.97 to 1.09, but other components and additives such as alcohols and esters have significantly lower response factors. The complexity of fuels makes the analysis of fuel exposures on a component-by-component basis impractical, and the toxicologic evaluation on a component-by-component basis impossible. And, since the composition of the liquid varies from lot-to-lot and day-to-day (due to

evaporation of the light ends), use of liquid fuel as a calibration standard does not permit comparison of results between laboratories and, therefore, further complicates industrial hygiene evaluations.

TABLE 3. FID RELATIVE RESPONSE FACTORS

Component	FID Response
Butane	1.09
Octane	0.97
2-methylpentane	1.05
Benzene	1.12
Toluene	1.07
Isopropanol	0.53
Octaldehyde	0.78
Isoamylacetate	0.62
n-Hexane	1.03

To provide a common analytical basis for comparison, we now calibrate our GLC-FIDs with n-hexane when analyzing jet fuels or other distillation-cut solvent exposure samples. All peak areas are integrated and the mass determined by multiplying by the FID response to n-hexane. The results are then reported as total hydrocarbons (THC) as n-hexane. Admittedly, the response of n-hexane may also be different than the response of the individual components (see Table 3), but n-hexane is a reproducible standard, is readily attainable, does not vary with time of storage, and has been historically used as a FID standard. If components of known toxicologic significance such as benzene or n-hexane are expected to be present, their presence is verified by mass spectroscopy (MS). MS identification is required because identification by retention time alone is unreliable.

The major problem we face is the interpretation of results. Field personnel have problems understanding results reported, "jet fuel, THC as n-hexane", and applying these results to a toxicologic exposure standard such as a Threshold Limit Value (TLV). Currently, we instruct personnel to apply the standards directly and to ignore the analytical calibration standard. While this is satisfactory as a first estimate, the universal use of n-hexane as a calibration standard will greatly facilitate the interpretation of toxicology data in industrial hygiene evaluations and will improve both inter-laboratory communications and the archival value of today's data bases.

As an additional consideration, several direct-reading instruments are now available such as portable FIDs and combustible gas indicators which can be calibrated with n-hexane and are used by fuel cell maintenance personnel to measure the THC concentration in

real-time prior to tank entry. Toxicity standards specified as THC as n-hexane would then be used to determine the respirator protection required to perform the maintenance task safely.

CONCLUSIONS

The evaluation of exposures to multicomponent, distillation-cut solvents such as jet fuels is a complex problem due to the difference between the vapor and liquid compositions and to batch-to-batch variations in the liquid composition. Such evaluations are further exacerbated by nonstandardized analytical procedures and standards for toxic exposures. To be truly useful for industrial hygiene evaluations, exposure standards need to be specified in terms of easily implemented analytical procedures. We recommend analyses for distillation-cut solvents and fuels be performed by gas liquid chromatography/flame ionization detector calibrated to n-hexane. Exposure standards will then be expressed as total hydrocarbons based on n-hexane as the calibration standard.

A SUGGESTED WORKPLACE MONITORING PROGRAM FOR MANAGING THE OCCUPATIONAL EXPOSURE OF U.S. AIR FORCE EMPLOYEES

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INTRODUCTION

The U.S. Air Force Occupational and Environmental Health Laboratory (USAF OEHL) has been assigned the responsibility of preparing draft Air Force Occupational Safety and Health Standards (AFOSH Standards), which are reviewed and modified by the Air Force Medical Service Center (AFMSC) prior to their final implementation within the U.S. Air Force. This paper will discuss the provisions of the draft AFOSH Standard 161-11, "Workplace Monitoring", prepared by Major Rock and Captain Lurker of the USAF OEHL, Consultant Services Division, Industrial Hygiene Branch. Currently, the document is in the review process. The contents of this paper reflect the policy in the proposed workplace monitoring program.

The draft AFOSH Standard 161-11 provides the following guidance:

- a. a formal strategy for prioritizing sampling surveys of USAF workplaces;
- b. risk assessment analysis of occupational health hazards to provide a formal priority system for line managers responsible for installing engineering controls; and
- c. statistical guidance to meet requirements of OSHA compliance sampling.

This paper concentrates on the first two aspects, the prioritization of sampling surveys and the Risk Assessment Code (RAC) analysis of the occupational health hazards. Toxicology information is a prerequisite for the hazard severity assessment of chemical and physical agents present in the workplace. As such, it is incorporated in each aspect.

BACKGROUND

Workplace monitoring procedures are necessary to efficiently allocate the limited resources available at a base and to maximize the probability that significant workplace deficiencies will be identified and evaluated. Once a survey has been completed, analytical results received and exposure levels calculated, a RAC procedure is needed to prioritize identified deficiencies so that commanders can efficiently allocate their limited operation and maintenance (O&M) resources to maximize the probability that more serious workplace deficiencies will be corrected first. Both the sampling priority and RAC analysis procedures were adapted from the "Assessment of Penalties" strategy outlined in the Occupational Safety and Health Administration (OSHA) Industrial Hygiene Field Operations Manual.

DECISION LOGIC OF PROPOSED STANDARD

Figure 1 illustrates a decision logic flowchart that describes the proposed workplace monitoring program. A presurvey is completed to collect necessary data for the prioritization of the survey schedule (this will be discussed in more detail later in the paper). The higher priority workplaces are surveyed first and then the survey measurements and analytical results received from the laboratory (usually USAF OEHL) are converted to exposure levels.

Briefly, before proceeding, I should clarify the difference between a survey measurement and an exposure measurement. A survey measurement refers to the raw analytical results. The exposure level measurements refer to data, including one or more survey measurements, that have been converted to Time Weighted Average (TWA) [8-hour or Short-Term Exposure Limit (STEL)] concentration. The exposure level measurements are to be compared to the appropriate AFOSH standard or OSHA Permissible Exposure Limit (PEL) or ceiling concentration standards.

Exposure level measurements are used in a RAC analysis (to be discussed later in this paper). As seen in Figure 1 (lower right side of the diagram) if a RAC of greater than 3 is determined, no further action is required by the base bioenvironmental engineer (BEE). For a RAC less than or equal to 3, the BEE must verify that the functional manager of the assessed workplace has filed an AF Form 3 with the unit safety office. These forms are retained by the unit safety office and information from them presented at the quarterly occupational safety and health meeting for commander notification and action. This RAC calculation is the single most important result of the workplace monitoring program, for no identified workplace deficiency will be corrected by a line manager without a sufficiently high priority on the list of outstanding construction projects. AFR 127-12, Air Force Occupational Safety

and Health Program, specifies that all safety- or health-related workplace deficiencies should be prioritized on the basis of the RAC.

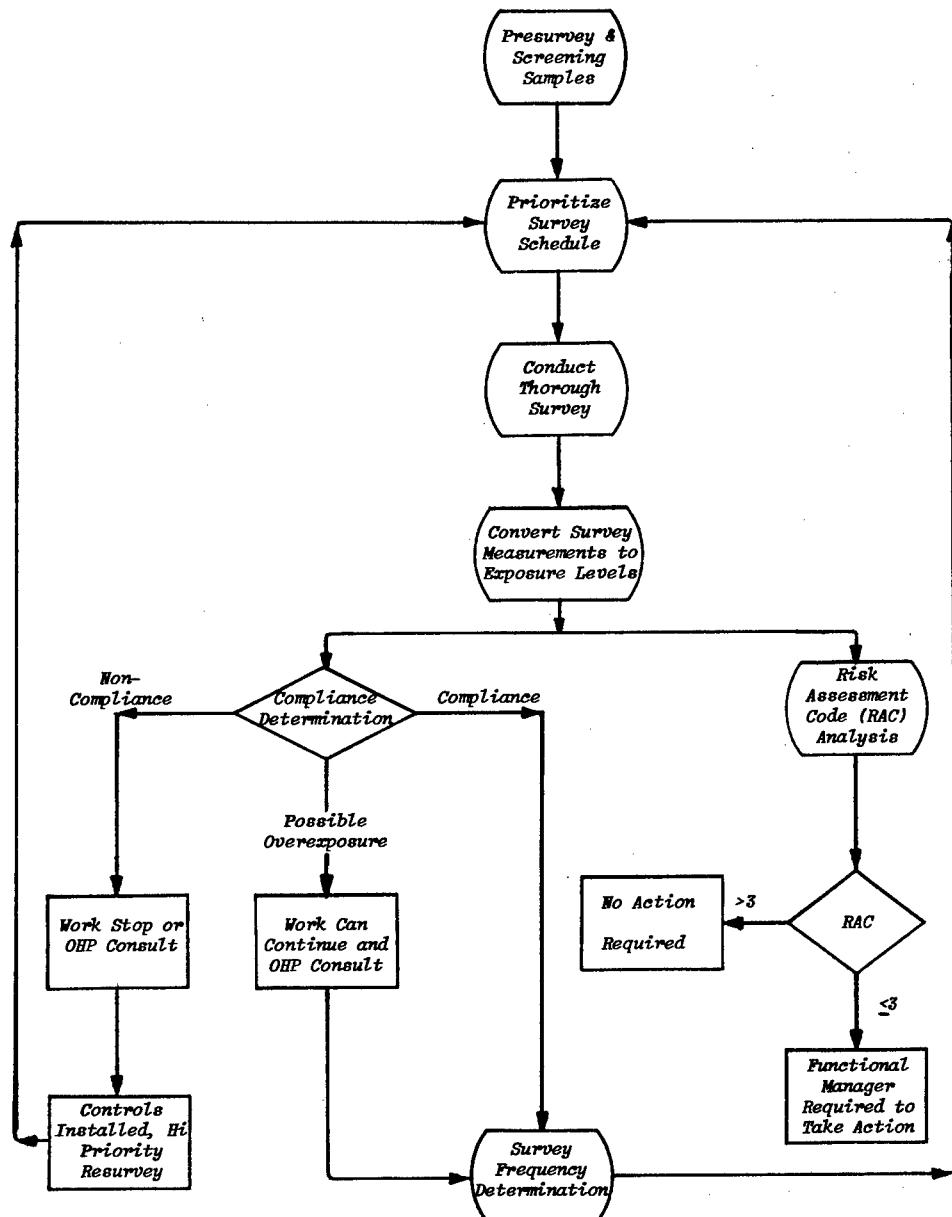


Figure 1. Decision logic flowchart for managing the workplace. OHP refers to an occupational health physician recommended medical monitoring requirement.

On the lower left side of Figure 1, the exposure levels are compared to the PELs and a compliance determination is made. To close the loop, a survey frequency determination is made and the survey requirement is placed in the survey schedule for prioritization (see top of Figure 1).

PRIORITIZING THE SURVEY

Attachment 1, (see Appendix) taken from the draft AFOSH Standard 161-11, contains instructions for completing a proposed AF Form, Sampling Priority Assessment Rating.

To schedule a survey, a priority rating assessment consisting of a probability quotient and a severity quotient must be calculated.

The probability quotient, as seen in Attachment 1, is a numerical assessment of the probability of illness based on the number of workers exposed, the use of personal protective equipment, an evaluation of the medical surveillance program, and a factor called "other considerations".

Attachment 1 also illustrates how points are determined for each factor. It should be pointed out that the medical surveillance factor is evaluated only when there is a feasible biological screening test. The BEE consults with the Aerospace Medicine Council (AMC) for its guidance. The BEE then uses a subjective opinion to determine if the medical surveillance program is adequate to effectively protect the workers.

The other considerations factor, as seen in Attachment 1, was adopted to introduce three types of survey requests:

- a. response to a specific complaint;
- b. new industrial process; and
- c. sampling required by directive.

If the BEE knew that the survey request was in response to a known occupational-health hazard that caused an illness or injury, an 8 point assessment is assigned to this other considerations factor.

If the other considerations factor is assessed 8 points, the priority rating is automatically assigned an 8 regardless of the value of the severity quotient.

To determine the probability quotient, see the equation presented in Attachment 1.

The severity quotient is the numerical assessment of the degree or the severity of illness (how sick someone could become) that can occur if workers are exposed to a particular agent. Fortunately, OSHA has classified the toxicity of most substances that have an OSHA standard in their Industrial Hygiene Field Operations Manual.

This manual provides a Substance Toxicity Table with a health code number that classifies the health hazard and can be used to determine the severity quotient. For nonlisted substances, base personnel will have to consult with the USAF OEHL. Recently, within the last year, a toxicologist, Capt Drawbaugh, was assigned to the USAF OEHL. The USAF OEHL will have to work with personnel at the AFAMRL/TH to obtain guidance on chemicals unique to the Air Force.

The priority rating, as seen in Attachment 1, is the average of the probability quotient and the severity quotient. It will range from 0 to 8 points. An assessment of 8 for the priority rating implies a high priority survey is required. The last page of Attachment 1 is a copy of a proposed AF Form, "Sampling Priority Rating Assessment". This form shows a sample calculation of the priority rating of a paint spraying operation.

RISK ASSESSMENT CODE (RAC) ANALYSIS

Attachment 2 (see Appendix), also taken from the draft AFOSH Standard 161-11 provides guidance for determining the RAC for occupational health hazards. Attachment 3 (see Appendix) explains how to complete a proposed AF Form, "Sampling Priority Rating Assessment". The last page of Attachment 3 provides a sample form with an example RAC calculation for a paint hangar.

As previously mentioned, the RAC is a relative determination to be used as a management tool to establish priorities for corrective action (primarily engineering controls). The RAC must never be used as an accurate numeric assessment of the individual health risks.

A hazard severity component is computed based on the potential effect of the chemical or physical agent on the body. It combines the medical effects factor (as seen in Attachment 2 is the toxicity of the agent) and the level of exposure factor. The points for each factor are combined and a Hazard Severity Category determined.

The mishap hazard component is the likelihood that existing environmental conditions will cause a mishap. It combines two factors, number of workers exposed and duration of exposure. Attachment 2 shows how the points are assigned for each factor. The total points are combined and the mishap probability category determined.

To determine the RAC, as seen in Table 5 of Attachment 2, a matrix has been developed. The Mishap Probability Category is the column entry and the Hazard Severity Category is the row entry.

ADDITIVE EFFECTS

The toxicity information required for this proposed workplace monitoring program is used in both the prioritizing of surveys as the severity quotient and in the RAC analysis as the medical effects factor. Both the severity quotient and medical effects factor depend on the available toxicology information to determine the severity of response to an agent. In addition to single agent assessment, the draft AFOSH Standard 161-11 gives guidelines on multiple agent exposure in the workplace. Initially, the BEE has to classify the multiple agents action as additive, independent, synergistic, potentiating or antagonistic. The background in toxicology of most BEEs is severely limited and it is expected that most BEEs will ignore the multiple agent guidelines. The American Conference of Governmental Industrial Hygienists (ACGIH) Threshold Limit Value (TLV) booklet specifies that, unless known to the contrary, assume that agents are additive. Therefore, BEEs will contact the USAF OEHL for guidance. However, information on multiple agent exposure is severely limited. Therefore, USAF OEHL personnel providing consultant services, especially our occupational health physician and toxicologist, must be aware of known multiple agent interaction. They must have access to a toxicology consultant service which we at the USAF OEHL hope will be available from AFAMRL/TH.

CONCLUSIONS

I've presented a proposed prioritization of survey strategy and a RAC analysis method. The document is still in the review process.

In closing, the intent of the AFOSH Standard 161-11, "Workplace Monitoring", is to reduce the occupational health hazards of the U.S. Air Force workplace in a cost effective manner.

APPENDIX

ATTACHMENT 1

INSTRUCTIONS FOR COMPLETING AF FORM XXXX, Sampling Priority Assessment Rating

The purpose of this form is to evaluate a workplace for sampling priority. The form allows calculation of the priority rating for sampling and provides documentation for why values were assigned to each factor considered.

1. Identification Data. Plastic embossed cards for recording identification data may be used in lieu of the following handwritten entries:

- a. Workplace Identifier (WI). Enter code for WI.
- b. Base. Enter name of base where workplace is located.
- c. Organization. Enter name of organization.
- d. Workplace. Enter name of workplace.
- e. Building Number/Location. Enter building number or location.
- f. Room/Area. Enter specific part of workplace being evaluated. If sample pertains to entire workplace, enter "NA" (not applicable).

2. Compounds or Physical Agents Evaluated. Specify the compound(s) or physical agents being evaluated. If compounds have dissimilar toxic action or collection media, prepare separate sampling priority rating assessment sheets.

3. Probability Quotient. For each factor evaluated, enter the basis and value of points assigned.

- a. Number of workers exposed:

Assign	
o 1 or 2 workers	1 or 2 points
o 3 to 6 workers	3 to 6 points
o 7 or more	7 or 8 points
- b. Duration of exposure:

o 1 to 8 hours per week	1 to 3 points
o over 8 hours per week but not continuous daily exposure	4 to 7 points
o continuous daily exposure	8 points
- c. Use of appropriate personal protective equipment (PPE):

o PPE is utilized by all exposed employees and a good program is in effect	1 to 2 points
o PPE is utilized by some of the exposed employees and minor deficiencies exist in the program	3 to 6 points
o PPE is not utilized by any of the exposed employees	7 to 8 points
- d. Evaluation of medical surveillance program: NOTE: If there is no applicable feasible biological screening test, this factor will not be considered. The BEE/EHT should consult with the Aerospace Medicine Council for guidance (ref AFR 161-33, para 1-11). This evaluation requires subjective analysis of the adequacy of the

medical surveillance program and how effectively it "protects" the worker.

- o effectively protects the employee 1 to 2 points
- o partially protects the employee 3 to 6 points
- o does not effectively protect the employee or is absent when it should be present 7 to 8 points

e. Other Considerations: Select the appropriate paragraph (1), (2), (3)

	<u>Assign</u>
(1) Response to a Specific Complaint	
o Physician Request for Consultation (SF 513)	7 to 8 points
o USAF Hazard Report (AF Form 457)	4 to 8 points
o A suspected overexposure (AF Form 190)	4 to 8 points
o Workplace where personnel have a high or increasing incidence of illness or injury	7 to 8 points
o Union or Employee Complaint	2 to 8 points
(2) New Industrial Process	4 to 8 points
o It is recommended to screen new processes as soon as possible, especially to determine potential health hazards. If all health hazards are known and are not serious	0 to 4 points
o If screening samples indicate exposures are below the AL	0 to 4 points
o If screening samples indicate exposures are between the AL and STD	4 to 6 points
o If screening samples indicate exposures exceed the STD	7 to 8 points
o If no screening samples exist or if there are unknown health hazards	8 points
(3) Sampling required by directive, such as AFOSH Standards, Air Force Regulations, technical orders and environmental differential pay or workmen's compensation directive.	

- o Exceptional mitigating circumstances such as good training, warning signs and labels, special procedures, or effective engineering controls, 0 to 2 points
- o Suspicious circumstances such as temporary maintenance problems with engineering control systems, poor training, or shortage of personal protective equipment, 4 to 8 points
- o If noncompliance has been determined for a previous survey of the workplace and RAC has not been assigned or is 1, 2, or 3, and RAC is 4 or 5, 6 to 8 points
- o If possible overexposure has been determined for a previous survey of the workplace and RAC has not been assigned or is 1, 2, or 3, and RAC is 4 or 5, 6 to 8 points
- o 4 to 6 points

NOTE: If the "other considerations" factor is assigned an assessment of 8, then the priority rating will also be assigned an 8 regardless of the assigned value of the severity quotient.

f. To determine the probability quotient, total the number of points assigned to each factor and divide by the number of factors used.

Probability quotient = [workers exposed factor + duration eval factor + PPE eval factor + med surv eval (if applicable) + other considerations factor] x

1

Number factors evaluated

4. Severity Quotient. Review the health hazards of the agent. Assign the Severity Quotient based on the severity classification (see paragraph 3b for severity classification). Enter the basis of the classification and assign points as follows:

Assign

- o If the classification is other than serious (o), 0 points

- o If the hazard can produce temporary, reversible illness requiring only supportive treatment, 1 or 2 points
- o If the hazard can produce temporary, reversible illness with a variable but limited period of disability, 3 to 6 points
- o If the classification is serious (s), or if the hazard can produce permanent, irreversible illness or health effects, 6 to 8 points

5. Priority Rating. Complete the priority calculation based on the formula $1/2$ (probability quotient + severity quotient) and enter the value.

6. Strategy. Check whether screening or compliance sampling is to be used. Select the sampling method, individuals to be sampled, sampling location(s), and number of samples needed. Record the collection media by referring to the USAF OEHL Recommended Sampling Procedures for the appropriate number.

7. Evaluated by. Record your name, grade, AFSC, sign and date.

(A handwritten example of the completed form is on the next page)

SAMPLING PRIORITY RATING ASSESSMENT		Workplace Identifier	0241//PACC//009A
		Base	Stratmberg AFB Organization
		Workplace	Paint Hangar
		Bldg No./Location	112
		Room/Area	N/A
Compounds or Physical Agents Evaluated			
Hexavalent Chromates (Paints)			
PROBABILITY QUOTIENT			
FACTOR (and point range)	BASIS	PTS. ASSIGNED	
NUMBER OF WORKERS EXPOSED			
1-7 Workers points = no., workers 7 Workers	5 Workers	5	
DURATION OF EXPOSURE			
1-8 hrs/wk ≥ 8 but < continuous continuous exposure	1-3 points 4-7 points 8 points	~ 6 Hrs/wk	
PERSONAL PROTECTIVE EQUIPMENT			
Effective	1-2 points	NIOSH Approved	
Minor Defects	3-6 points	Paint - Spray Respirators	
None	7-8 points	Used	
MEDICAL SURVEILLANCE			
Effective	1-2 points	Aerospace Med. Council Specified	
Minor Defects	3-6 points	Annual Exam w/o Recommended	
Major Defects	7-8 points	Chest x-ray, periodic Liver, blood Ref. NIOSH Occup. Guide To Disease	
OTHER CONSIDERATIONS		P9-354	
Response to Specific Complaint	2-8 points	Directive Sampling	
New Industrial Process	4-8 points	No. Engineering Controls	
Directive Sampling	0-8 points	(No Ventilation System)	
Total Pts. 4			
NOTE: IF OTHER CONSIDERATIONS = 8 pts, then Priority Rating = 8 pts			
PROBABILITY QUOTIENT = TOTAL POINTS/NUMBER OF FACTORS = 19/5 3.8			
SEVERITY QUOTIENT			
FACTOR (and point range)	BASIS	PTS ASSIGNED	
SEVERITY CLASSIFICATION			
Other than serious (o)	0 pts	Suspect Carcinogen	
Temp. Reversible Illness-Minor Rx	1-2 pts	Ref: OSHA IH FOM	
Temp. Reversible Illness-Disability	3-6 pts	Chap II Table.	
Permanent Irreversible Illness	7-8 pts	8	
SEVERITY QUOTIENT = POINTS ASSIGNED =		8	
$\text{PRIORITY RATING} = \frac{1}{2} (\text{Probability Quotient} + \text{Severity Quotient})$ $= \frac{1}{2} (3.8 + 8) = 5.9$			
STRATEGY for Screening & Compliance			
SAMPLING METHOD	INDIVIDUAL SAMPLED		
<input type="checkbox"/> Full Period Consecutive	<input type="checkbox"/> Full Period Single		
<input type="checkbox"/> Partial Period Consecutive	<input checked="" type="checkbox"/> Batch Process		
<input type="checkbox"/> Grab	<input type="checkbox"/> Swipe		
SAMPLING LOCATION(S)	NO. OF SAMPLES	COLLECTION MEDIA	
<input type="checkbox"/> Backgd.	2	PV 50-434	
<input checked="" type="checkbox"/> Breathing Zone	<input type="checkbox"/> Source of Emission		
EVALUATED BY (Name, Grade, AFSC)	SIGNATURE	DATE (YYMMDD)	
Grady, P.B. JLT, 9121A	Paul B. Grady	810621	

Attachment 1. Sampling Priority Rating Assessment Example

ATTACHMENT 2

GUIDANCE FOR DETERMINING RISK ASSESSMENT CODES (RACs)
FOR OCCUPATIONAL HEALTH HAZARDS

Risk Assessment Codes (RAC) provide a systematic method of prioritizing resources to correct health and safety hazards. As outlined in AFR 127-12, two components (hazard severity and mishap probability) are used to determine the RAC. Determination of these components depends upon knowing the toxicity of the contaminant or the effect the agent may have on the body, the concentration or intensity of the contaminant or agent in the workplace, the exposure time, and the number of workers exposed.

DETERMINING HAZARD SEVERITY:

The hazard severity due to occupational health hazards is determined from two factors, severity of the exposure and effects of exposure. Consult Table 1 and complete the Hazard Severity Section of AF Form XXXX, the Risk Assessment Code Calculation Sheet as described in Attachment 3.

TABLE 1. HAZARD SEVERITY FOR HEALTH HAZARDS*

(1) Medical Effects

Condition	Points
Violation of administrative requirements such as posting of warning signs, nuisance noise, control of nuisance odors	0
Exposure may result in temporary, reversible illness requiring supportive treatment	1 to 2
Exposure may result in temporary, reversible illness with a variable but limited period of disability, or permanent, non-severe illness or loss of capacity (i.e., permanent hearing loss)	3 to 6
Exposure may result in permanent, severe irreversible illness or death	7 to 8

(2) Level of Exposure

Condition	Points
Measured or estimated inhalation exposure level below the Action Level and skin absorption or ingestion not possible	0
Measured or estimated inhalation exposure level below Action Level, skin absorption or ingestion possible	1 or 2

TABLE 1 (cont'd)

Measured or estimated inhalation exposure levels occasionally exceed Action Level but Permissible Exposure Level (PEL) not exceeded and skin or ingestion not possible	3
Measured or estimated inhalation exposure levels occasionally exceed Action Level but PEL not exceeded, skin absorption or ingestion possible	4
Measured or estimated inhalation exposure level exceeds Action Level but PEL or Short-Term Exposure Level (STEL) not exceeded and skin absorption or ingestion not possible	5
Measured or estimated inhalation exposure levels exceed Action Level but PEL or STEL not exceeded, skin absorption or ingestion possible	6
Measured or estimated inhalation exposure levels exceed PEL or STEL but skin absorption or ingestion not possible	7
Measured or estimated inhalation exposure levels exceed PEL or STEL, skin absorption or ingestion possible	8
Average of all applicable measurements exceeds PEL or STEL	9

*In Table 1, assign points on the basis of the UCL. That is, if the UCL of the measured or estimated exposure exceeds one, assign the approximate number of points

TABLE 2. ASSIGNING HAZARD SEVERITY CATEGORY

<u>Hazard Severity</u>	<u>Total Points</u> [Sum of (1) + (2)]
Category I	13-17
Category II	9-12
Category III	5-8
Category IV	0-4

DETERMINING MISHAP PROBABILITY

Mishap probability is the likelihood that the existing environmental condition will produce a mishap. The mishap probability for health hazards is determined by two factors, the number of

workers exposed and duration of exposure. Consult Tables 3 and 4 and complete the Mishap Probability Section of AF Form XXXX, Risk Assessment Code Calculation Sheet as described in Attachment 3.

TABLE 3. MISHAP PROBABILITY FOR HEALTH HAZARDS

(1)	Number of Workers Exposed	Points
	Less than 5	1 to 2
	5 to 9	3 to 4
	10 to 49	5 to 6
	50 or more	7 to 8
(2)	Duration of Exposure	
	2 to 8 hours per week	1 to 3
	Over 8 hours per week but not continuous daily exposure	4 to 7
	Continuous daily exposure	8

TABLE 4. ASSIGNING MISHAP PROBABILITY CATEGORY

<u>Mishap Probability</u>	<u>Total Points (1) + (2)</u>
A	14 to 16
B	10 to 13
C	5 to 9
D	Less than 5

The Risk Assessment Code (RAC) is determined from the following matrix:

TABLE 5. RISK ASSESSMENT CODE MATRIX

		# Mishap Probability			
		A	B	C	D
Hazard	I	1	1	2	3
Severity	II	1	2	3	4
Category	III	2	3	4	5
	IV	3	4	5	5

The RAC should be recorded on the AF Form XXXX, Risk Assessment Code Calculation Sheet, as described in Attachment 3.

RISK ASSESSMENT CODE CALCULATION SHEET (Reference Atch 2, AFOSH Std 161-11)		Workplace Identifier 012411 F/A/C/C/0094
		Base Strombeag AFB Organization 26 FMS
Workplace Paint Hangar		
Bldg. No./Location 117		Room/Area NIA
HAZARD SEVERITY		
Medical Effects IRREVERSIBLE ILLNESS	Summary Hexavalent Chromium (Suspect Carcinogen) Present During Paint Spraying Operations	PTS ASSIGNED 7
Level of Exposure Environmental Measured Levels Exceed AFOSH STD Skin Adsorption is Possible	Summary Max. Environmental Level = 82 $\mu\text{g/m}^3$ TLV = 50 $\mu\text{g/m}^3$ No ventilation system but approved Respirators P.E. = 10X	PTS ASSIGNED 8
		TOTAL POINTS 15
HAZARD SEVERITY CATEGORY (See Table 2 of ATCH 2, AFOSH Std 161-11): T		
MISHAP PROBABILITY		
Number of Workers Exposed: 6	Assigned Pts.	
Less than 5	1 to 2	
5 to 9	3 to 4	
10 to 49	5 to 6	
50 or more	7 to 8	Number of Workers Exposed Points: 3
Duration of Exposure : 4 to 6 Hrs/wk		
1 to 8 hours/week	1 to 3	
>8 hrs/wk but not continuous	4 to 7	
continuous daily exposure	8	Duration of Exposure Points: 3
		TOTAL POINTS 6
MISHAP PROBABILITY CATEGORY (See Table 4 of ATCH 2, AFOSH Std 161-11): C		
RISK ASSESSMENT CODE (RAC) (See Table 5 of ATCH 2, AFOSH Std 161-11): 2		
ACTION RECOMMENDED: (Attach sheet(s) if necessary)		
Ventilation System is recommended in lieu of respirators.		
ACTIONS TAKEN 30 DAYS AFTER BEING REPORTED: (Note: AFR 127-12 para 24 a (3) specifies that if the RAC is a 1, 2 or 3 that an AF Form 3 must be initiated by the functional unit.		
26 FMS Submitted AF Form 3 on 81 09 02 to Wing Safety.		
EVALUATED BY (Name, Grade, AFSC)	SIGNATURE	DATE (YYMMDD)

Attachment 2. Risk Assessment Code Calculation Sheet Example

RISK ASSESSMENT CODE CALCULATION SHEET (Reference Atch 2, AFOSH Std 161-11)		Workplace Identifier Base	02411 F A C C 0094 Stramberg AFB 26 FMS	Organization
Workplace Bldg. No./Location		Paint Hangar 117 NIA		
HAZARD SEVERITY				
Medical Effects IRREVERSIBLE ILLNESS	Summary Hexavalent Chromium (suspect carcinogen) present during Paint Spraying operations		PTS ASSIGNED 7	
Level of Exposure Environmental Measured Levels EXCEED AFOSH STD Skin Adsorption is possible	Summary Max. Environmental Level = 82 μ g/m ³ TLV = 50 μ g/m ³ No ventilation system but approved respirators P.F. = 10X		PTS ASSIGNED 8	
TOTAL POINTS				15
HAZARD SEVERITY CATEGORY (See Table 2 of ATCH 2, AFOSH Std 161-11):				
MISHAP PROBABILITY				
Number of Workers Exposed: 6	Assigned Pts. 1 to 2 3 to 4 5 to 6 7 to 8		Number of Workers Exposed Points: 3	
Duration of Exposure : 4 to 6 Hrs/wk 1 to 8 hours/week >8 hrs/wk but not continuous continuous daily exposure	1 to 3 4 to 7 8		Duration of Exposure Points: 3	
TOTAL POINTS				6
MISHAP PROBABILITY CATEGORY (See Table 4 of ATCH 2, AFOSH Std 161-11):				
RISK ASSESSMENT CODE (RAC) (See Table 5 of ATCH 2, AFOSH Std 161-11):				
ACTION RECOMMENDED: (Attach sheet(s) if necessary) Ventilation system is recommended in lieu of respirators.				
ACTIONS TAKEN 30 DAYS AFTER BEING REPORTED: (Note: AFR 127-12 para 24 a (3) specifies that if the RAC is a 1, 2 or 3 that an AF Form 3 must be initiated by the functional unit. 26 FMS Submitted AF Form 3 on 81 09 02 to Wing Safety.				
EVALUATED BY (Name, Grade, AFSC)	SIGNATURE		DATE (YYMMDD)	

Attachment 3. Risk Assessment Code Calculation Sheet Example

ATTACHMENT 3

INSTRUCTIONS FOR COMPLETING AF FORM XXXX,
Risk Assessment Code Calculation Sheet

The purpose of this form is to provide a calculation sheet to determine the Risk Assessment Code required for correction of an occupational health hazard in a workplace. The form provides documentation for why a particular hazard severity and mishap probability was assigned and a record of recommended actions.

1. Identification Data. Plastic embossed cards for recording identification data may be used in lieu of the following handwritten entries:
 - a. Workplace Identifier (WI). Enter code for WI.
 - b. Base. Enter name of base where workplace is located.
 - c. Organization. Enter name of organization.
 - d. Workplace. Enter name of workplace.
 - e. Building Number/Location. Enter building number or location.
 - f. Room/Area. Enter specific part of workplace being evaluated. If sample pertains to entire workplace, enter "NA" (not applicable).
2. Hazard Severity
 - a. Medical Effects. Refer to Table 1, Attachment 2, for a list of medical effects and determine the classification that best describes the occupational health effects of the workplace under evaluation. Give a brief summary of the basis for the medical effects classification selected. Assign the appropriate points and record the points in the assigned column.
 - b. Level of Exposure. Refer to Table 1, Attachment 2, for a list of level of exposure. Determine the proper classification and record the points in the assigned column.
 - c. Total the points for medical effects and level of exposure and record as total points. Refer to Table 2, Attachment 2, for a Hazard Severity Category and record this category on the form.

3. Mishap Probability
 - a. Number of Workers Exposed. Record the number of workers exposed and assign the appropriate points based on Table 3, Attachment 2.
 - b. Duration of Exposure. Record the estimated duration of exposure the workers receive per week. This estimate is based on the workplace schedule and administrative controls that have been implemented. Assign and record the appropriate points based on Table 3, Attachment 2.
 - c. Total the points for number of workers exposed and duration of exposure and record the total points. Refer to Table 4, Attachment 2, for a mishap probability category and record this category on the form.
4. Risk Assessment Code (RAC). Consult the RAC matrix in Table 5, Attachment 2. The row entry of the matrix is the hazard severity and the column entry of the matrix is the mishap probability. Find the proper row and column entry and record this RAC (1 to 5) value on the form.
5. Evaluated By. Record name, grade, AFSC, sign and date (YY MM DD).
6. Actions Recommended. On the form describe the recommended action. Attach additional sheets if necessary.
7. Actions Taken 30 Days After Being Reported. IAW AFR 127-12, para 24a(3) if the assigned RAC is 1, 2 or 3, an AF Form 3 must be completed by the line manager responsible for the workplace and submitted to the unit safety office.

THE DEVELOPMENT OF A COMPUTERIZED DATA BASE TO ENHANCE APPLIED AND THEORETICAL TOXICOLOGY

Major Robert A. Lombard, Jr., USAF, BSC

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Brooks Air Force Base, Texas

The Air Force has been conducting an aggressive occupational health program for over 30 years. The goal of that program is to protect the worker - both military and civilian - by providing a healthful work environment that is free of recognized chemical, physical, or biological hazards. The successful accomplishment of this goal is an essential contributor to our overall military readiness.

Failure to provide a safe environment can affect the worker in two general ways. First, short-term acute illnesses can cause unacceptable losses in productivity. Second, and more insidious, are long-term chronic exposures that cause gradual impairments such as hearing loss. When these effects are discovered, the Air Force not only loses the worker's productivity but also the experience base the worker has developed over many years - a commodity very difficult to replace.

There are also financial ramifications to worker health. Air Force illness and injury costs have risen to \$62.4 million for worker compensation and \$1.9 million for environmental differential pay in 1980. Attempts to reduce illness and injury through engineering control of hazards is also expensive. The cost to correct just serious Air Force deficiencies has risen to \$360.5 million (in 1980).

Finally, increased impetus to occupational health is provided by recent changes to documents which implement the Occupational Safety and Health Act of 1970. They include Executive Order 12196, 29CFR1960, DOD Instructions 6055.1 and 6055.5, and DOD Manual 6055.5. These significantly increase program work load because of more complex administrative requirements.

COMPREHENSIVE OCCUPATIONAL HEALTH PROGRAM

These factors have prompted a new approach to occupational health referred to as the Comprehensive Occupational Health Program. The new program builds on existing effects, requiring a mix of MFP-6 (research and development) and MFP-8 (operational) activities. The overall program can be broken down into six functional elements or subprograms. They are:

1. Standardized Occupational Health Program (SOHP)/Computerized Occupational Health Program (COHP). Standardization of the current USAF Occupational Health Program includes significant upgrading of worker and workplace data documentation. The manually gathered data base will then be automated in the Computerized Occupational Health Program (COHP). Both of these subprograms will be discussed in detail later in this paper.
2. Detection Equipment Capabilities and Technologies (DETECT)/Protective Equipment Capabilities and Technologies (PROTECT) Programs. These elements will address the evaluation and selection of sampling procedures and instrumentation (DETECT), and personal protective equipment (PROTECT) unique to Air Force industrial workplaces.
3. Toxicologic Research and Epidemiology of Noise and Dangerous Substances (TRENDS)/Computerized Assessment of Radiation Exposures (COMPARES) Programs. These will address toxicologic responses to dangerous chemical substances and the physiologic responses to noise and radiation. Human exposure data will be coupled with animal toxicology study results for the purpose of retrospective and prospective epidemiology.

SOHP/COHP and DETECT/PROTECT are operational programs being developed by the USAF Occupational and Environmental Health Laboratory. TRENDS and COMPARES are future research capabilities that will be available to the Air Force Aerospace Medical Research Laboratory and the USAF School of Aerospace Medicine, respectively.

The complexity of the program elements has required a multi-phased, long-term approach. SOHP has been developed in Phase I. The automated aspects - COHP - are now under development as Phase II of the comprehensive program. Once a COHP data base has been established, Phase III - the initiation of DETECT, PROTECT, TRENDS, and COMPARES - can begin. Since the ultimate success of the comprehensive program depends greatly on COHP and its manual predecessor, SOHP, the rest of this paper will address their capabilities.

STANDARDIZED PROGRAM (SOHP)

Before development of the standardized program began, the weaknesses of existing occupational health activities at base level were examined. Workplace information, stored in case files, was found to be too descriptive and input oriented. It had insufficient concrete information, such as exposure concentrations, and was very difficult to retrieve. There was little standardization of data entry and no uniform way of identifying workplaces. Information gathered on workplace exposures was not transferred to the health records of exposed workers to enable cause and effect relationships to be identified. Finally, nearly all aspects of base level worker/workplace surveillance were done manually. This resulted in slow response to any inquiries and prevented even simple attempts at trend analysis. Recordkeeping was manpower intensive and served to divert scarce manhours from the principal tasks of worker/workplace surveillance.

SOHP was developed to eliminate or minimize many of these weaknesses. The case files have been reoriented to enable rapid retrieval of information and a standard workplace nomenclature, called the workplace identifier, has been developed. Twenty-one standard data forms have been developed. Seventeen are for recording industrial hygiene workplace data in the case files. Three are for clinical occupational health examination data in the worker's health record. The twenty-first form is the Master Workplace Exposure Data Summary, a form used to transfer exposure data from the case file to the worker's health record. An Air Force Occupational Safety and Health Standard, AFOSH STD 161-17, has been written to implement these solutions. It is currently undergoing review by the Air Staff.

In spite of these improvements, the program will still be conducted in a manual mode, preventing many useful applications of the data at base level, by major air command and Air Staff, and by the research community. A computerized program is therefore needed.

COMPUTERIZED PROGRAM (COHP)

COHP must satisfy the needs of many diverse customers ranging from base level bioenvironmental engineers and health care providers to research scientists. A key requirement for base level users is real-time access. Cathode ray tubes (CRTs) must be capable of displaying worker/workplace data instantly if the information is to be useful in health risk decision making. From the major air command and Air Staff viewpoint, COHP must feature an occupational health management information system (OHMIS), that is, up-to-date summaries of work load, discrepancies, and exposure data that indicate the status of base programs and enhance policy decision making. Comprehensive, statistically

accurate data must be output to enable operational and research laboratories to obtain feedback from base level activities. Finally, existing automated data bases, such as the USAFSAM Hearing Conservation Repository, must be networked with COHP to effectively utilize all available data, both past and present.

These features have been incorporated into the COHP specifications. Data, displayed on CRTs and printed on line printers, will be in the same "forms" mode as the standardized program. In this way, the information will be directly usable without the assistance of computer specialists. Access will also be user oriented through the extensive use of menu screens to move about the programs and prompted data screens to minimize typing and associated errors.

The first of four program segments, Industrial Hygiene Chemical Exposure Surveillance Data, is now being developed. Portions are also being field tested via a remote terminal at the Randolph Air Force Base Clinic. Future software segments will be Industrial Hygiene Physical/Biological Exposure Surveillance Data, Clinical Occupational Health Surveillance Data, and Environmental Surveillance Data.

Each segment provides automated enhancement of the entire processes. For example, industrial hygiene surveys can be scheduled and necessary data forms printed. When the survey is completed, data are entered into the computer and stored. Calculations and library searches can be made to enable decision making and these decisions will be automatically printed in reports. Major air commands will be able to retrieve preprogrammed management summaries of exposure data while epidemiologists can rapidly search thousands of records to compare exposure concentrations to affected workers examination results under varying environmental conditions.

THE COMPREHENSIVE MODEL

SOHP and its automated version, COHP, can be viewed as tools that enable occupational surveillance data to flow from base level through the major air commands to a central computer repository. From this central point, information is fed back to base level and is also provided to the Air Staff, operational laboratories and the R&D community as demanded.

The linch-pin of the entire process is COHP. Its development and gradual activation throughout the Air Force will take five years. Resources involved total \$10.3 million as five year life cycle costs. Because of the user oriented features, only nine additional manpower spaces will be required to manage the central data base and hardware.

SUMMARY

SOHP and COHP are on schedule. AFOSH STD 161-17 has been completed and tested at seven Air Force bases. It is now under Air Staff review. The Data Automation Requirement for COHP is being submitted and software development is underway. If resource requirements are supported, the initial development of COHP will commence at ten bases in fiscal year 1983. This will be followed by additional bases added each year through fiscal year 1988.

The Air Force goal is to provide a healthful work environment that is free of recognized chemical, physical, or biological hazards that can cause death or serious physical harm. This goal can only be achieved with a comprehensive, multiphased approach incorporating operational and research program elements. A vital part of this approach is the efficient flow of occupational health information to and from all professionals involved. This flow will be significantly enhanced by the Computerized Occupational Health Program.

OPEN FORUM

DR. CROCKER (University of California, Irvine): I certainly welcome the session this morning as a link between field observations and the toxicology laboratory and I see that industrial hygienists are developing a very valuable additional tool. The epidemiologist whose uses of these data from both the toxicologic and the field exposure records point of view will be in a much better position to assess risk. The integrated point of view of the overall program of assessment of hazard to personnel should direct the attention of the toxicologist as to what issues are of greatest importance at the military base level stands as a very fine advance. I think as representatives of the University, we see this as the kind of integration that we ourselves are trying to make in our occupational health program which as a part of our other activities includes our concern for the same issues in industry in our own area. We see ourselves attempting to establish similar patterns of recording worker hazard, including even setting up categories of worker function, that is, the various types of work that are done and trying to arrive at some kind of standardized approach to the degree of hazard each worker has so that we can begin to draw categories of hazard for the various functional occupational assignments. I think it would be very interesting to consider compounds that we can envision as becoming important in the near future, and ask ourselves to the degree to which that compound would fulfill any of the assessments of risk, the degree to which there is adequate evidence of widespread exposure or likelihood of exposure and the degree to which the toxicologic data are available now to support your needs or where more data may be needed. In other words, another kind of a pilot level effort which would be a seat-of-the-pants attempt to look at a single compound and see how that might fall into this line of assessment and integration of data achievement or data recording. I could think of a candidate compound, fluomine, for example. Is this oxygen storage agent likely to be in effective use in a future time; it has been anticipated, toxicologic studies have been done but the anticipation of its actual usage we don't know. But it has some very interesting toxicologic features. As toxicologists, we'd like to know if this were pursued further from the point of view of the user, which is yourselves and the workers whom you protect. Is this a candidate compound for that kind of integration of effort? Hydrazine has already had a very nice workup in that direction where the toxicologic data were effective, extensive, where field operation potential for exposure was genuine and where an estimate of the real hazard to the worker could be made in terms of how serious a carcinogenic risk it is. We already have some examples of case by case combinations of effort. I'd like to see us continue to do this with new compounds in parallel with the development of this very effective recording system.

MAJ. LOMBARD (U. S. Air Force Occupational and Environmental Health Laboratory): Your point is definitely well taken. We emphasize, of course, the base level pilot study mainly because it's a cart and horse situation and of course the inputs have to come before the outputs. You're absolutely right; once we have demonstrated the feasibility of getting reasonable data into our system, we then have to insure that the data are indeed of value both to the managers and to the epidemiologists and toxicologists. In our program we envision a liaison stage in our development in which some of these questions will be answered and in which we would in effect do many pilot studies on the output side of things to be sure that we don't spend an awful lot of time at the military base level gathering data that you don't want. That, indeed, would be a waste of all our resources.

DR. SLONIM (Air Force Aerospace Medical Research Laboratory): I just want to say to Dr. Rock that I think his approach looks very sound. My question is, how new is it and how much exposure has it had amongst your peers? In other words, how accepted is it amongst your fellow industrial hygienists?

LT. COL. ROCK (U. S. Air Force Occupational and Environmental Health Laboratory): The part of the approach that deals with setting of standards is receiving its first public exposure this morning. It will receive its second public exposure two weeks from today at a conference of setting standards sponsored by the joint Army/Navy/Air Force/NASA Sub-Committee on Environmental Science. The rest of the material describing the nature of occupational exposures and the nature of occupational exposure measurements has been presented at national Industrial Hygiene conferences since 1979 in various stages. It has triggered enough interest that there's a small research group in the Northeast and another one at Berkeley that are pursuing it and expanding it beyond the limits of my knowledge. Unfortunately, none of the work is funded at this point. It seems that there just isn't any formal mechanism for funding the basic research necessary to form the basis for rational decisions in this area.

MR. VERNOT (University of California, Irvine): I'd like to add my expression of admiration for Col. Rock's approach to developing a rational method for the gathering and utilization of industrial hygiene data. It did appear to me that there was one point where the use of sound statistical and engineering principles could no longer be used in setting standards and in utilization of the data and that seemed to be right at the start when a decision was made, if I understood properly, that unacceptable work limits were going to be determined by saying that it was unacceptable to have more than 30% of the measured points over the 95% confidence limits of the STEL and that the maximum probable exposure that was deemed to be unacceptable was four times the limit. My question is, what criteria did you use to set those particular standards?

LT. COL. ROCK (U. S. Air Force Occupational and Environmental Health Laboratory): The standard on the long-term average exposure is a proposal of mine that serves as an example of an idealized standard. It's not one that's really useful in practice because to apply that standard, you must know with pretty good confidence the variability of the measurements that you're dealing with. And with the three or four, sometimes seven or eight, measurements that we typically have available, we don't have high confidence that we know the variability of the workplace. However, it's worth looking at the behavior of your decision criteria if you assume that all the assumptions that are implicit and sometimes not stated in the development of those criteria are met, how do they behave? That's what I was looking at. That's not to say that it's the way it behaves in practice, but this is the way it's designed to behave; the two charts where I showed the maximum probable exposure for the OSHA and the legal action level are more how it behaves in practice. You do not control the maximum probable exposure, and, indeed, if you look at the real world and know nothing of statistics, the maximum probable exposure is when the valve seat ruptures or the pipeline breaks or the worker falls in the vat that's got a catwalk around it and the maximum probable exposure is way out there on the tail and it's very difficult to control those. I'm not sure that answers your question entirely.

MR. VERNOT: I just wondered why you picked the standard of four times the limit for a maximum probable exposure as being the dividing line between acceptable and non-acceptable.

LT. COL. ROCK: The reason I picked that value is because the old ACGIH Threshold Limit Value booklet, until about four years ago, used to say that any time the exposure excursions exceeded three times the standard during an eight-hour period even though the time weighted average value was below the standard, that was unacceptable in the least hazardous of situations. The other excursion factors were 2 and 1.5 in the more hazardous situations. And the one that I chose is rather arbitrary, but I say I'll let an excursion in a full eight-hour exposure go to four times the standard before I'll say that that workplace is just unacceptable; that we ought to do something about it from a management perspective by redesigning the process or implementing administrative controls or something. If we mean anything at all when we set the standards, then four times the standard is too much.

DR. TORKELSON (Dow Chemical U.S.A.): I have a comment for Dr. Lurker. I saw no indication that there was any credit for doing things right. For example, there was no credit for education of your workers. And I think that this is probably the most valuable thing the industrial hygienists can do is to be sure the workers are trained and knowledgeable about what they're handling. I do have a serious caveat and that is the epidemiologists

are going to have to be extremely careful. In our experience, the gathering of medical data in multiple locations has just brought on all sorts of problems. One place will use a particular respirometer carefully; another place will operate a respirometer at a different temperature and there is never an effort to get these two instruments in agreement. They have not been used in the same way; in one place there's been somebody interested in respiratory physiology and he's kept careful control on it; in the other place they've been using it strictly as a screening method. If your methods are not standard and instruments are not calibrated properly, you're going to find out you have one Air Force Base that appears to be sick and the rest of them will look healthy and that's what the data say. I think you're going to have to build into your system something to protect you from bad data reaching the epidemiologist.

MAJ. LOMBARD (U. S. Air Force Occupational and Environmental Health Laboratory): Your point is very well taken. In fact, it's even worse than that! We have found that even in simple standardized wet chemistry type procedures each individual laboratory, of course, has its norms and these norms are highly variable from one medical laboratory to another. We are trying to tackle that problem and you understated, I think, just how big a problem that may be.

COL. MacNAUGHTON (Air Force Aerospace Medical Research Laboratory): I have an inherent distrust of large data bases because of the general lack of quality control that goes into them. How do you propose that the data be checked at some point along the line and that it is, in fact, correct? And the other question that I always have is who really has access to the data base and how are you going to protect personal information? You say you will have information such as someone's grandmother has had an abortion or something like that. Who really has access to that information and how is it protected by the laws of the country and how does the Freedom of Information Act affect this information?

MAJ. LOMBARD (U. S. Air Force Occupational and Environmental Health Laboratory): I guess there are actually two questions here; how do we QC the data, first of all. We do it in a number of ways. One is by reducing the need to QC the data and we do that mainly through this user oriented system. We found that the more you can design a computer system to resemble the normal way of doing business, the better off you are. We found another interesting thing with computers you can take a 17 or 18 year old and he does just fine with a computer terminal because he's used to playing Star Wars but older people make all kinds of errors. So there is an education problem in QC. We have, in the design of our software, put in limiting parameters which serve as our second line of defense on the quality control of the data. There is also a review of the data that arrive at the central host

computer which we now envision to be located at the OEHL at Brooks Air Force Base. That kind of leads us into your second question on the security of the information. There are actually two sides to the security thing; I'm surprised that the gentleman from Dow didn't ask what are we doing about the proprietary information on the chemical constituents of some of their products. Because that is, to some, just as important as the Privacy Act side of protecting medical data. We have two things that we're using to protect information. First of all, we have the normal security systems, both the physical security systems such as locked doors and that type of stuff and physical barriers in our main data base and that already exists because we have some sensitive data already. We have another thing, however, that is being designed for our military base level which I think is probably the place where you could get more abuses because there are more different people using the data. There will be a heirarchy of information accesses. For example, a technician would require one level of access, an engineer in a position would require yet another level and someone in an administrative capacity would require yet another level of access. So we're protecting both our proprietary chemical information and our medical data in those ways.

DR. DEETER (U. S. Army Environmental Hygiene Agency): I have two questions. Major Lombard, how does the system you describe compare with the Army's proposal for OMUS and do you have your seven and one half million dollars?

MAJ. LOMBARD (U. S. Air Force Occupational and Environmental Health Laboratory): How does it compare to OMUS? OMUS is concentrating, to my knowledge, on the inventory side of things. It is shooting for an earlier implementation than we are and I think that's an admirable thing. We will not have even close to complete information for five years and that's the price we're paying. OMUS will have it much sooner because it is strictly inventorying the status quo as it exists right now. It is a batch oriented system, it is not real time and I guess you could call that the negative side to OMUS. Our system eventually will be more useful on a day to day basis. I don't think OMUS is designed with the same goals in mind and may be more useful in a centralized location than ours. I should mention that the Navy has some excellent ideas that they're working on for computerized occupational health systems. Now, as to the real important question, where's the 7.2 million dollars? We now have pretty firm indications that we have approximately 50% of that money lined up. We are fighting just as everyone else does for resources. We are relatively optimistic that we can get our total funding. If we do not, we do have some contingency plans for reduced scope systems but we hope that doesn't happen because we'd like the entire flow to take place from base level through batch communications to a central host and most important, to some of the users such as yourselves.

DR. DEETER (U. S. Army Environmental Hygiene Agency): Captain Lurker, I can appreciate your attempts to quantify a decision making process to when we should monitor workplaces or survey individuals. In several of your categories, I noticed there was a lot of area for subjective decisions to be made to assign a number for your system. If these subjective decisions have to be made to give a number, how is this different from what we're doing already?

CAPT. LURKER (U. S. Air Force Occupational and Environmental Health Laboratory): Currently in the determination of when to conduct a survey, the base industrial hygiene personnel have to consider the politics involved in the situation. For example, the union is demanding a survey for environmental differential pay and we immediately respond to that request, but that doesn't particularly protect workers from occupational health exposures and subjectively, the industrial hygienist could assign direct and lower assessment points for doing that survey or he could decide in that subjective factor that he will give it higher points based on the politics. And your point is well taken in some parts of the strategy, that the subjective factor will probably take politics into consideration.

DR. DEETER (U. S. Army Environmental Hygiene Agency): Colonel Rock, I appreciate the system for basing standards on a reasonable approach and you mention several places where the standards should be based on biological halflife, total body burden that's tolerated in association with airborne concentration and that makes a lot of sense. I'm sure we would like to do that. What I would ask, sir, is where do we get those data?

LT. COL. ROCK (U. S. Air Force Occupational and Environmental Health Laboratory): That's why I made the proposal to this group. We would hope to get this information from the toxicologist.

DR. HENDERSON (Olin Corporation, Retired): I am concerned that personal eating habits cannot be completely recorded and that the nutritional status and the recreational activities of the worker may influence his overall health. You obviously can go to great lengths and great expense to establish and control a work environment at a no-effect, perfectly healthful level. And yet this young woman of child-bearing age that you are so carefully protecting for eight hours a day goes home and in her garage she refinishes furniture with unknown solvents and paint strippers; she makes stained glass windows with lead levels that are considerably higher than anything allowed in industry or the work environment and she comes back to work the next day sick. I think it's going to buy us our information badly. I hope these problems can be overcome because the system that is being developed would be so beneficial to us in R and D that I think it's worth the effort.

CAPT. LURKER (U. S. Air Force Occupational and Environmental Health Laboratory): It is labor intensive to manually calculate the percentage of recommended dietary allowances when we've got 45 identified and you may have 40 to 45 individual food items in three meals in a day so you've got about 1600 calculations and summation to get that, but there are also computer programs where you can input by common food name and get a computer printout of the percent of RDA and you might want to look at these. Again, it only gives you an approximation because you start with a diary of food intake. But this is something that is being developed that can give you a nutritional profile much easier than we used to be able to do it. I've used a four-day diary, Saturday, Sunday, Monday, Tuesday, and then input the data into the computer. The one that I've been using presently only has 20 of the essential nutrients, but it is being programmed to give the essential amino acids, the polyunsaturated as against the saturated fats, and some of the others. We hope to have that up to the total essential nutrients eventually. So you might be interested in some of that because it does give you a much better nutritional profile than we've ever been able to get before.

SESSION IV

APPLICATIONS OF PHYSIOLOGICAL KINETIC MODELING IN TOXICOLOGY

Chairman

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**PHARMACOKINETIC EVALUATIONS IN RELATION TO USE OF
TOXICOLOGICAL DATA**

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INTRODUCTION

In order to provide a sound scientific basis for recommending acceptably safe levels of human exposure to potentially harmful chemicals, toxicologists are confronted with the problem of projecting laboratory animal toxicity data to the expected response in humans. Chronic toxicity studies in animals are conducted at dose levels that are usually many orders of magnitude higher than those encountered under environmentally realistic conditions. Furthermore, different mammalian species may be either more or less sensitive on a body weight basis to the toxic action of a given chemical. Therefore, prediction of potential hazard is a two-dimensional problem in that it involves not only extrapolation from laboratory animal species to humans but also extrapolation downward from experimental dose levels to those encountered in the environment. Pharmacokinetic principles comprise an essential component of the entire array of toxicologic considerations that must be utilized in achieving a realistic projection of the expected toxicologic risk to humans. Application of pharmacokinetic principles can provide information aiding in both the interspecies and the high to low dose extrapolations of toxicity data. This paper discusses in particular the impact of the transition from linear to nonlinear pharmacokinetics upon the dose versus response curve.

DOSE-RESPONSE AND INTERSPECIES RELATIONSHIPS

The complex problem of extrapolating toxicology data may be envisioned in the diagram of Figure 1a in which the chemical dose level (on a body weight basis), the species size (or body weight), and the incidence (or severity) of the toxic response all increase in an outward direction from the coordinate intersection. The height of the surface above the dose-species plane in the absence of a chemical dose represents the normal background incidence of a

given toxic response. The shaded area in the region of the higher dose levels in Figure 1a shows the experimentally observable range of the dose versus response relationship in smaller animal species. The arrows indicate the directions of extrapolation of toxicity data across the toxicity surface in order to arrive at an estimate of the potential risk to humans at realistic exposure levels.

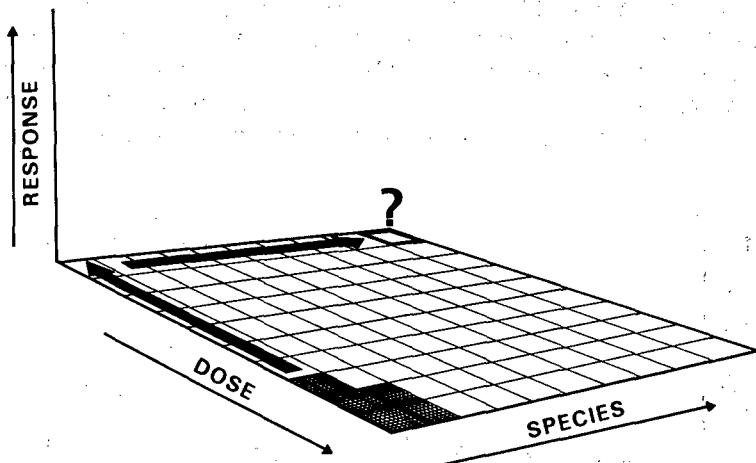


Figure 1a. A 3-dimensional representation of the dose-response-species interrelationship. All quantities increase in an outward direction from the coordinate intersection.

When a toxic response is elicited by a chemical, the dose-response curve can be envisioned to lie in the vertical dose-response plane for a given species as shown in Figure 1b. As the dose level decreases, the toxic response also diminishes until it virtually vanishes into the background incidence of the lesion induced by the toxic agent.

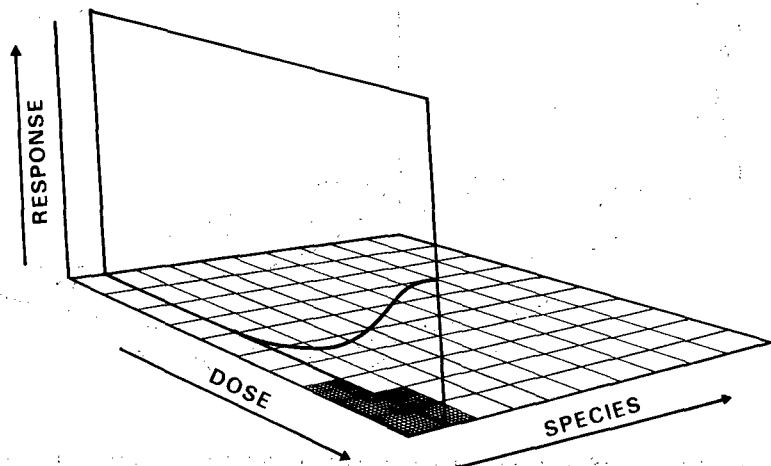


Figure 1b. A dose-response curve for a given species (lying in the vertical dose-response plane).

At a given dose level the toxic response may either increase or decrease as the species size increases, and this response can be envisioned to lie in the vertical species-response planes of Figure 1c and 1d, respectively. Since the basal metabolism rate of different mammalian species is approximately proportional to the body surface area-to-volume ratio and this ratio increases with decreasing body size, small animals will in general metabolize chemicals more rapidly on a body weight basis than larger animals. Therefore, whether a larger animal species will be more or less sensitive to a chemical than a smaller species is often dependent on whether metabolism of the parent chemical constitutes a detoxication or an intoxication process, respectively.

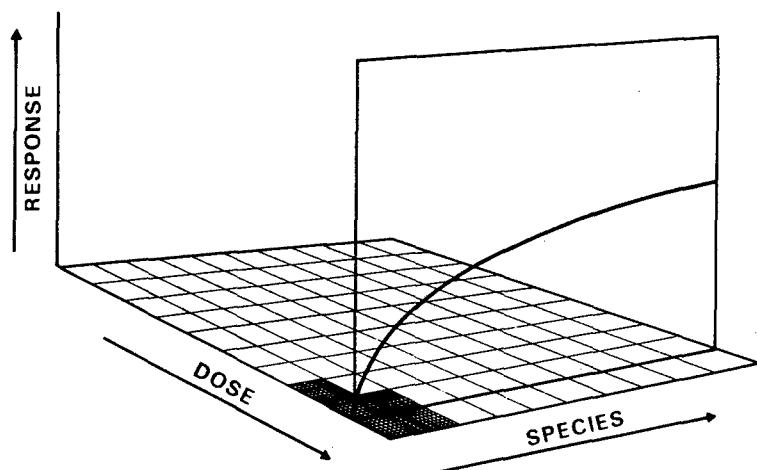


Figure 1c. A species-response curve increasing with increasing species size at a given dose level (lying in the vertical species-response plane).

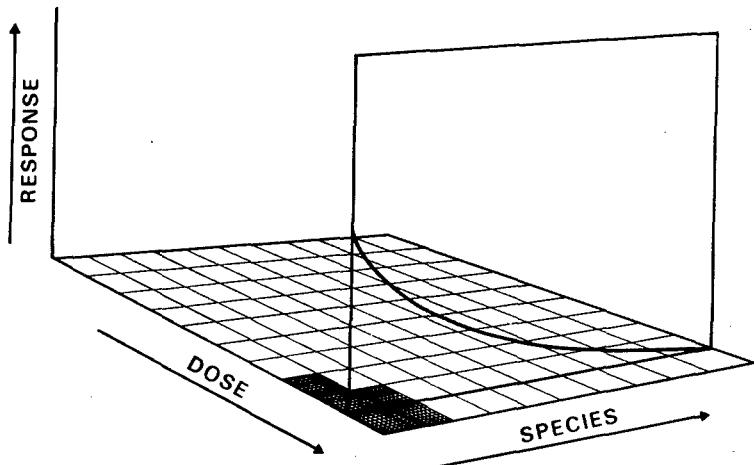


Figure 1d. A species-response curve decreasing with increasing species size at a given dose level (lying in the vertical species-response plane).

The foregoing considerations imply that the toxicity surface lying above the plane of Figure 1a may easily assume a complex shape between the extremes of dose level and species size, and that the shape of this surface is quite likely different for different chemicals. Studies elucidating the predominant mechanism of toxicity of a given chemical can reveal directional trends across this surface as both species and dose levels change. Likewise, a knowledge of the pharmacokinetic profile of a chemical can provide both qualitative and quantitative information concerning the expected toxic response with changing dose levels in different species (Ramsey and Gehring, 1980; Watanabe et al., 1980). The complex nature of the surface emphasizes the necessity of integrating the different disciplines of toxicology and utilizing all available information in a logically consistent manner in order to obtain the most realistic estimate of the potential risk to humans.

PRINCIPLES

Pharmacokinetics is the discipline that studies the dynamics of absorption, distribution, metabolism, and excretion of a chemical in the body. Pharmacokinetic models are mathematical constructions that quantify these processes, and are usually expressed as a set of simultaneous differential equations which describe the rates of change of the model variables. Compartmental models usually infer the existence of kinetically similar groups of tissues from the shape of the blood concentration versus time curve, and these models are often capable of accurate mathematical descriptions of the data (Gibaldi and Feldman, 1969; Levy et al., 1969; Wiegand and Sanders, 1964). On the other hand, physiologic models utilize physiologic and anatomic parameters in conjunction with the observed data (Lutz et al., 1977; Himmelstein and Lutz, 1979). While they are usually more complex than classic compartmental models, physiologic models may more adequately explain kinetic nonlinearities (Andersen, 1981; Andersen, 1979), as well as provide a rational basis for interspecies extrapolation of pharmacokinetic data (Dedrick, 1973; Dedrick and Bischoff, 1980).

Since pharmacokinetic models quantitate the internal concentrations of a parent chemical and its metabolite(s) as a function of time and as a function of dose level, a pharmacokinetic model can relate the internal concentration of toxicant to the dose level of a chemical.

Many of the physiologic and biochemical processes incorporated in pharmacokinetic models such as active transport and enzymatically mediated biotransformations are capacity limited (i.e., saturable). Other processes such as mass transfer across permeable membranes and glomerular filtration may exhibit kinetically linear properties over a much wider range of substrate concentrations. However, when any rate-limiting physiologic or biochemical

process involving the absorption, distribution, metabolism, or excretion of a chemical becomes saturated the pharmacokinetic profile will exhibit properties of kinetic nonlinearity.

The Michaelis-Menten rate equation presented in the inset of Figure 2 is often utilized in pharmacokinetic models; it is capable of representing a range of relationships from approximately first order rates at chemical concentrations well below the K_m value to virtually saturated or zero order rates at concentrations substantially higher than the K_m value. The key significance of first order rates lies in the constant quantitative relationship maintained between reacting species when a system is controlled by first order kinetic processes, especially when a chemical is repeatedly or continuously administered. The gradual departure from the nearly linear (i.e., first order) portion of the rate curve in Figure 2 as the chemical concentration increases comprises the transition from linear to nonlinear pharmacokinetics. This transition is of fundamental importance, since at chemical concentrations above this transition region, the constant proportionality maintained at lower concentrations (i.e., lower dose levels) is lost. It is of primary concern in projecting toxicity versus dose level data that this transition region from linear to nonlinear kinetics be defined as a function of the applied (or external) dose level.

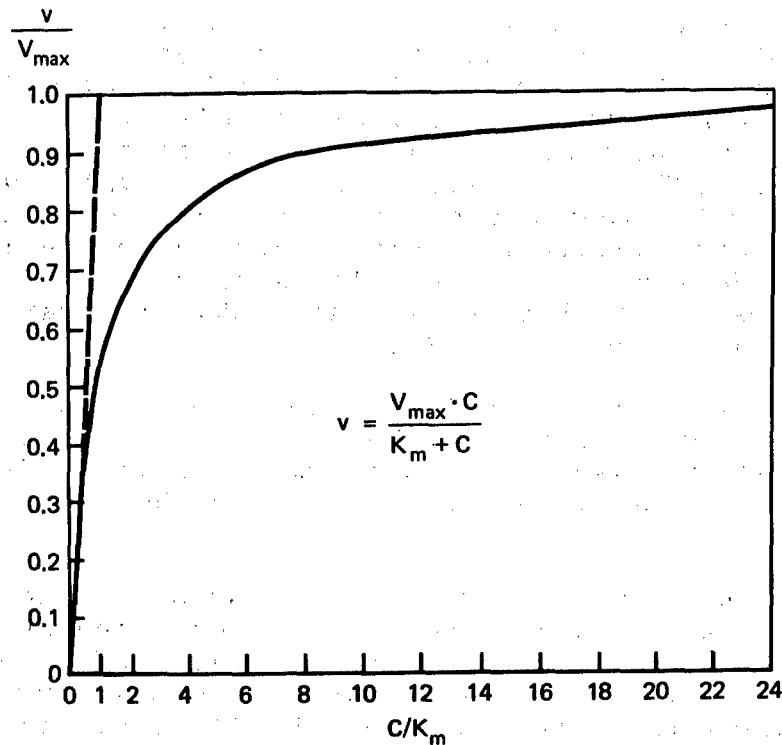
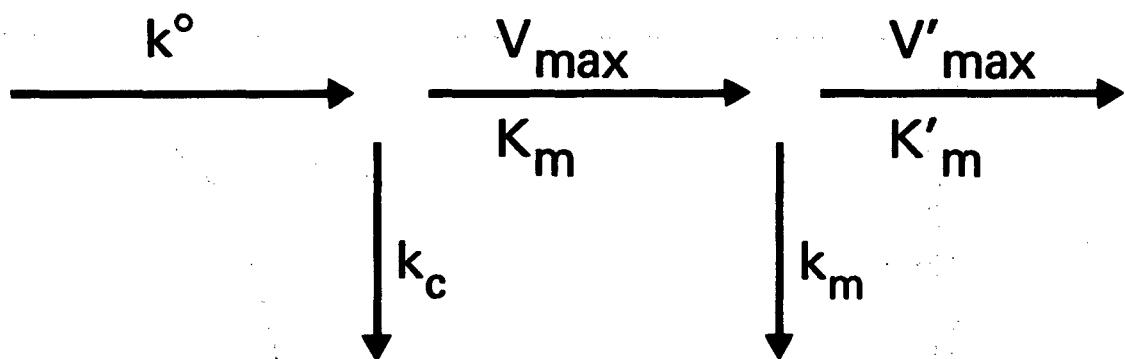


Figure 2. The normalized reaction rate versus concentration curve for a process obeying Michaelis-Menten kinetics. v =reaction velocity; V_{max} =maximum reaction velocity; C =reactant concentration; K_m =reactant concentration at $v=0.5V_{max}$ (the Michaelis constant); the dotted line is a projection of the first order portion of the rate curve.

HYPOTHETICAL ILLUSTRATION

The hypothetical model chosen to illustrate the impact of pharmacokinetic nonlinearity is presented in Figure 3. The dose rate (dose level) is considered to occur at an uninterrupted constant rate (k^o) as might be the case for continuous environmental exposure or for an intravenous infusion. The parent chemical C can be excreted from the body by a first order process K_c or transformed to metabolite M by the saturable process characterized by V_{max} and K_m . The metabolite can also be excreted by a first order process K_m' or further metabolized by a saturable process characterized by V'_max and K'_m .



C = concentration of parent chemical

M = concentration of metabolite

k^o = input (dose) rate

Figure 3. Hypothetical pharmacokinetic model describing the disposition of a chemical (C) and its metabolite (M) in the body. $V_{max} = 3.41 \mu\text{mole}/\text{hr}$; $K_m = 0.598 \mu\text{mole}$; $V'_max = 4.95 \mu\text{mole}/\text{hr}$; $K'_m = 10.9 \mu\text{mole}$; $k_c = 0.114/\text{hr}$; $K_m' = 0.015/\text{hr}$; k^o varied from 0.0001 to 300 $\mu\text{mole}/\text{hr}$.

Continuous input of the chemical will eventually result in attainment of steady state concentrations within the body of both the parent chemical and its metabolite. Since a toxic response under continuous exposure conditions will most likely be a function of the steady state concentration of toxicant, the steady state concentration can be considered as the effective or internal dose.

Biologically plausible values were chosen for the pharmacokinetic parameters of the model (see the legend of Figure 3), and the steady state concentrations of C (C_{ss}) and of M (M_{ss}) were determined by numerical integration of the differential equations describing the model (Ramsey and Reitz, 1980). These steady state concentrations of the chemical and its metabolite were determined at values of the dose rate ranging from 0.0001 to 300. In order to illustrate the relationship between the steady state values of C and M to the dose rate each value of C_{ss} and M_{ss} was divided by the corresponding dose rate and the results are shown in Figure 4.

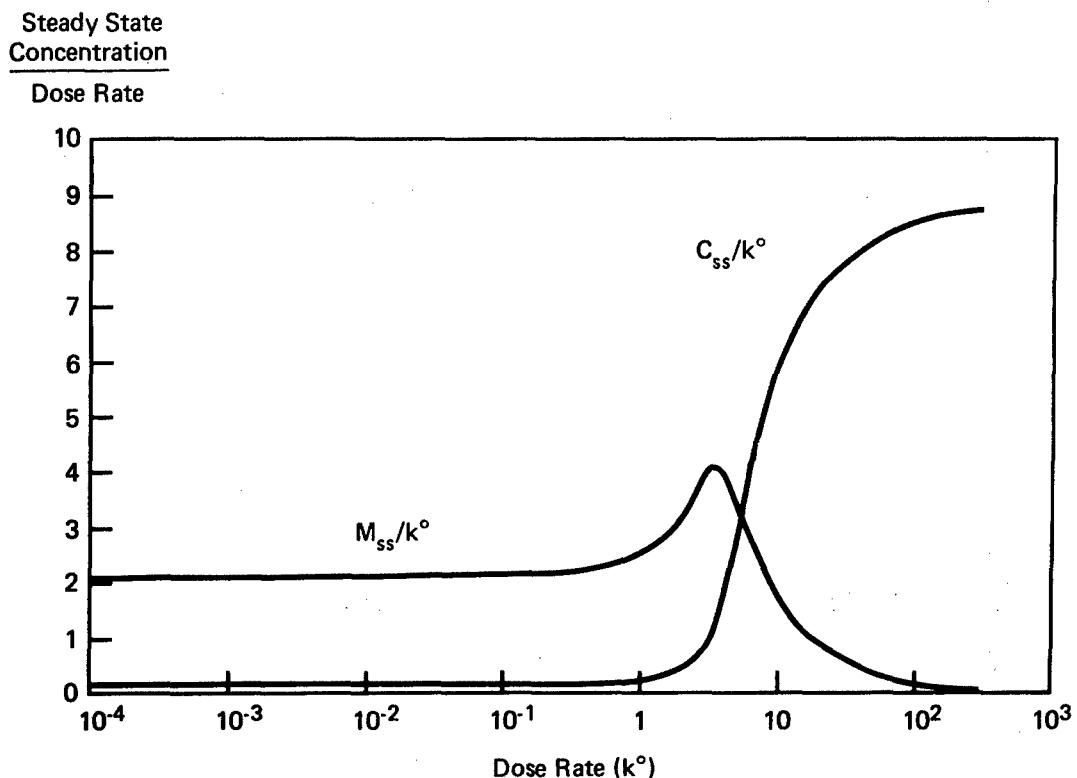


Figure 4. The ratio between steady state concentration of the parent chemical and dose rate (C_{ss}/k°), and between steady state concentration of the metabolite and dose rate (M_{ss}/k°). Calculated by numerical integration of the pharmacokinetic model in Figure 3.

Linear pharmacokinetics are indicated in this example at the lower dose levels where the ratios of C_{ss}/k° and M_{ss}/k° maintain a constant value. In other words, the linear properties of a first order kinetic system determine that the quantitative relationship between the internal steady state concentrations (C_{ss} or M_{ss}) and the dose rate (k°) remains constant. Thus, within the dose range

where the curves of Figure 4 remain nearly parallel to the abscissa, the steady state concentrations of the parent chemical and its metabolite in the body remain directly proportional to the dose rate.

However, kinetic nonlinearity becomes evident in this example as the dose level increases in the range of 0.1 to 1.0. As the concentration of parent chemical C approaches and then exceeds the value of the Michaelis constant for its metabolic transformation, a dramatic increase in the ratio of C_{ss}/k^o is evident. A concurrent increase in the ratio of M_{ss}/k^o is also apparent until the rate of formation of M from C becomes virtually saturated. At successively higher dose rates M_{ss} stays almost constant and consequently the ratio of M_{ss}/k^o decreases as the dose rate increases. Thus, at dose rates above the transition from linear to nonlinear kinetics the relationships between the dose rate and the steady state concentrations of the parent compound and its metabolite have been altered relative to the nearly constant relationships that are maintained below the pharmacokinetic threshold region.

The impact of this pharmacokinetic nonlinearity on a toxic response can be examined by choosing an arbitrary response function and calculating the toxic response that might result when either the parent compound or its metabolite is the toxic entity. The one-hit model for carcinogenic response is the most conservative of many such models, but it has the single virtue of simplicity, and is representative of all dose-response functions in its dependence on the internal concentration of the toxic entity (VanRyzin, 1980; Gehring et al., 1979). For illustrative purposes, the response R was calculated according to the one-hit model as $R=1-\exp(-\beta D)$ where the sensitivity factor β was set at 0.001 and the internal dose D was the steady state concentration of either C_{ss} or M_{ss} as previously calculated.

In this manner, the response R was calculated for the entire range of simulated dose rates. The resulting dose-response curves are plotted as the solid lines in Figure 5 (in which C is the toxic entity) and 6 (in which M is the toxic entity). The dose-response curve arising from the parent compound exhibits a nearly sigmoid shape with a response of 9.26×10^{-1} at a dose rate of 300 decreasing to 1.72×10^{-8} at a dose rate of 0.0001. The dose response curve arising from the metabolite shows a maximum response of 1.86×10^{-2} at the high dose rates, decreasing to 2.09×10^{-7} at a dose rate of 0.001. The flat portion of the response curve at the higher dose levels is a consequence of the saturable rate of formation of the metabolite, regardless of the increasing concentration of the parent chemical in the body.

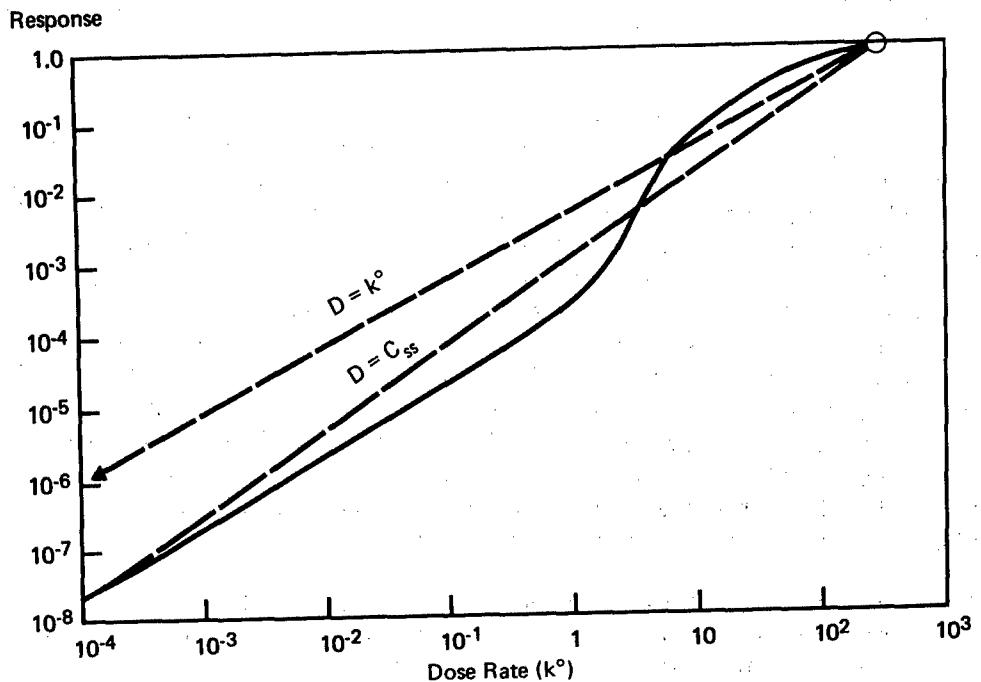


Figure 5. Dose-response curve (solid line) generated from the pharmacokinetic model of Figure 3 in which the parent chemical (C_{ss}) is the toxic entity and the response is calculated with the one-hit model. The dashed lines are explained in the text.

In practice the toxic response observed at an applied dose level D (utilizing the one-hit model) is used to calculate β from the relationship $\beta = (-\ln(1-R))/D$. This value of β is then used in the one-hit model equation given above to calculate the predicted response R at a lower value of the dose level D . In Figure 5, the dashed line labeled " $D = k^\circ$ " shows the response predicted at a dose rate of 0.0001 based on the observed response at a dose rate of 300 when the calculations are performed using the dose rate (k°) as D . The predicted response clearly overestimates the observed response. On the other hand, the dashed line of Figure 5 labeled " $D = C_{ss}$ " shows the correctly predicted response at a dose rate of 0.0001 based on the observed response at a dose rate of 300 when the internal dose (C_{ss}) is used rather than the dose rate in the foregoing calculations.

A similar analysis is presented in Figure 6 in which the response is accurately predicted when the extrapolation is based on the internal concentration of the toxicant (M_{ss}). On the other hand, when the prediction is based on the dose rate at doses above the transition from linear to nonlinear pharmacokinetics, the predicted response is clearly in error.

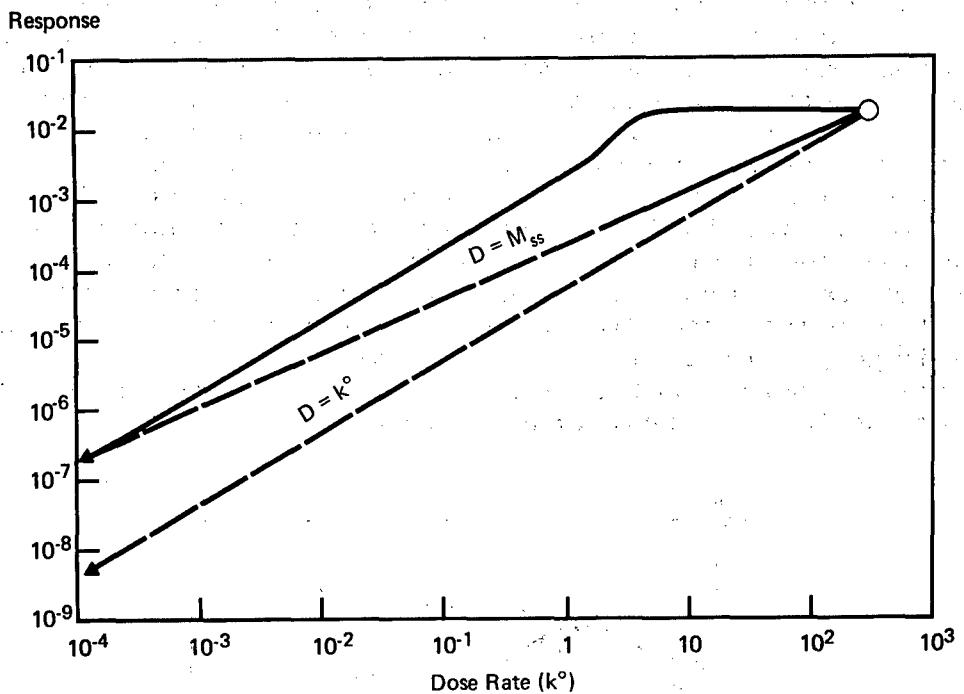


Figure 6. Dose-response curve (solid line) generated from the pharmacokinetic model of Figure 3 in which the metabolite (M_{ss}) is the toxic entity and the response is calculated with the one-hit model. The dashed lines are explained in the text.

This example demonstrates the inadequacy of dose-response data obtained at dose levels above the nonlinear pharmacokinetic region to predict the response at lower dose levels when the prediction is based only on the applied dose rate. However, adequate knowledge of the pharmacokinetic profile and the mechanism of toxicity enable a reasonable extrapolation to lower dose levels by indexing the calculations to the internal concentration of the toxic entity.

It is important to note that the foregoing example is an illustration only of the impact of a nonlinear kinetic profile upon the relationship between internal concentrations and dose level. While it is obvious that such phenomena must be considered in predicting a toxic response at very low dose levels based on the response at high dose levels, other highly important considerations can not be ignored. Thus, when natural defense mechanisms such as reaction with glutathione or DNA repair are responsible for mitigating the toxic response, they may be overwhelmed at sufficiently high internal concentrations of the toxicant, and thereby produce further nonlinearities in the actual dose-response curve (Watanabe et al., 1977; Gehring and Blau, 1977).

EXAMPLE

Styrene provides an example both of a transition from linear to nonlinear pharmacokinetics as the external dose level (or exposure level) increases and of a direct pharmacokinetic comparison between experimental animals and humans. Figure 7 presents the blood concentration versus time curve for styrene in rats that were exposed for 6 hours to 80 ppm, then removed from the inhalation chamber to determine the clearance kinetics, and also in rats exposed to the same concentration for up to 24 hours. The uptake and approach to an equilibrium concentration of styrene in blood was rapid and reached a steady state value of approximately 0.8 μg per ml after several hours. In those rats removed from the inhalation chamber at 6 hours, the clearance of styrene from blood exhibited the log-linear characteristics that are typical of a linear two compartment pharmacokinetic model in which all the rate processes are operating in the first order region.

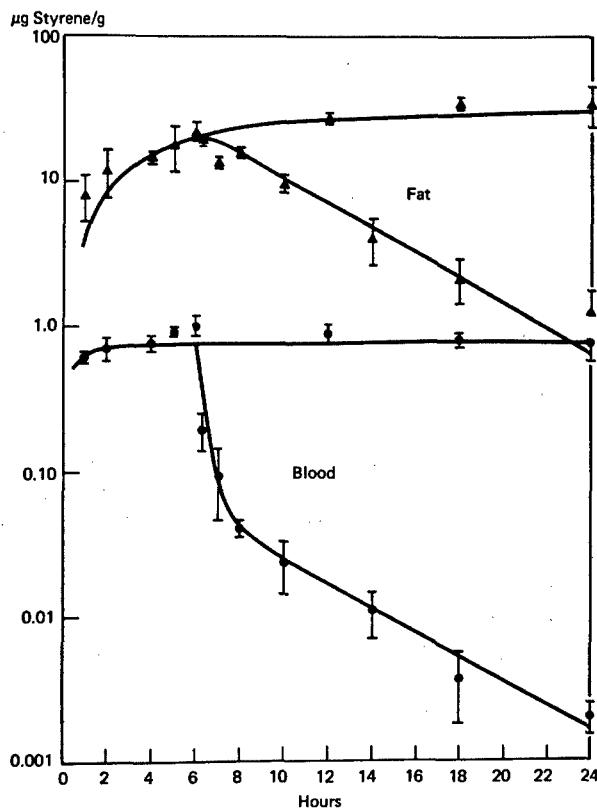
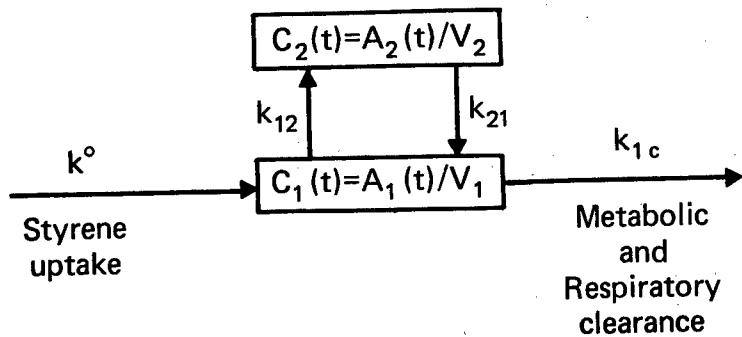


Figure 7. Concentration of styrene in blood and fat of rats exposed to 80 ppm for up to 24 hours. A group of rats was removed from the inhalation chamber at 6 hours to obtain the clearance data. Each data point is the mean \pm standard deviation for 3 rats. Solid lines are predicted concentrations calculated with the best pharmacokinetic parameter estimates of the model shown in Figure 8.

Figure 8 presents the linear two-compartment model that was used to characterize the uptake and clearance of styrene in rats at an inhaled concentration of 80 ppm, and the best pharmacokinetic parameter estimates of this model provided the theoretical concentration curves represented by the solid lines in Figure 7. The half life value of the terminal blood clearance phase was approximately 3.5 hours. In another group of rats exposed to 200 ppm styrene, the pharmacokinetic profile also exhibited linear kinetic characteristics. However, at 600 ppm the kinetic profile exhibited definitely nonlinear characteristics, and the maximum blood concentration attained was disproportionately high compared to that at 80 and 200 ppm. At an exposure concentration of 1200 ppm the nonlinear characteristics were even more pronounced, and the curves showing blood concentrations at this inhaled concentration are shown in Figure 9. Note that, while the exposure concentration was increased 15 fold from 80 to 1200 ppm, the maximum blood concentration was increased almost 80 fold. This is typical disproportionality in a pharmacokinetic variable that indicates the dose level has been increased sufficiently to evoke nonlinear kinetic behavior.



$$\frac{dA_1(t)}{dt} = \begin{cases} k^{\circ}, 0 < t \leq t^* \\ 0, t > t^* \end{cases} + k_{21} A_2(t) - (k_{12} + k_{1c}) A_1(t)$$

$$\frac{dA_2(t)}{dt} = k_{12} A_1(t) - k_{21} A_2(t)$$

Figure 8. Pharmacokinetic model and differential equations describing the uptake, distribution, and clearance of inhaled styrene in rats and humans. k° =zero order input rate; k_{12} and k_{21} =first order mass transfer coefficients; k_{1c} =apparent first order rate constant for combined metabolic and respiratory clearance; $C_i(t)$ =styrene concentration in compartment i at time t ; $A_i(t)$ =amount of styrene in compartment i at time t ; V_i =apparent volume of distribution of compartment i ; t =total elapsed time; t^* =time at end of exposure.

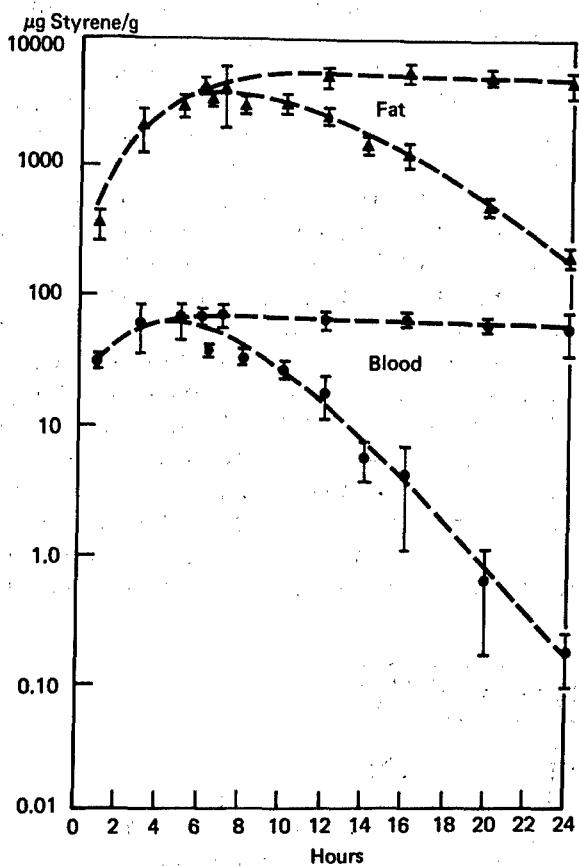


Figure 9. Concentration of styrene in blood and fat of rats exposed to 1200 ppm for up to 24 hours. A group of rats was removed from the chamber at 6 hours to obtain the clearance data. Each data point is the mean \pm standard deviation for 3 rats. Dashed lines were drawn by inspection.

This nonlinearity is further evidenced by a comparison of the areas under the blood concentration versus time curves (AUC) presented in Table 1 for the 4 exposure concentrations. Note that as the exposure concentration was increased 15 fold, the internal dose of parent chemical (measured by the AUC) was increased 95 fold. The linear (first order) properties of the pharmacokinetic model of Figure 8 are insufficient to explain the pharmacokinetic nonlinearity evidenced at the higher concentrations, since these disproportionately high blood concentrations are a consequence of the interaction of both saturable physiologic and biochemical kinetic parameters (Andersen, 1981). Regardless of the mechanism of saturation, it is most important here that there is a transition from linear to nonlinear kinetic behavior at concentrations exceeding approximately 200 ppm. Therefore, referring to the previous discussion, if toxicity were observed as a result of exposure to saturating (i.e., nonlinear) concentrations of styrene, these data could not be extrapolated to the expected response at lower concentrations based on the exposure concentration alone.

TABLE 1. AREAS UNDER BLOOD CONCENTRATION CURVES (AUC) 0-24 HOURS IN RATS EXPOSED TO 80, 200, 600 OR 1200 PPM STYRENE FOR 6 HOURS

Exposure Concentration (ppm)	AUC ($\mu\text{g}\cdot\text{hr}/\text{ml}$)	Normalized to 80 ppm	
		Exposure Concentration	AUC
80	5.8	1.0	1.0
200	8.6	2.5	1.5
600	160	7.5	28
1200	550	15	95

The pharmacokinetic behavior of inhaled styrene in human volunteers has also been studied at a concentration of 80 ppm (Ramsey et al., 1980). The blood concentration of styrene in humans is presented in Figure 10, and is seen to be very similar to that observed in rats at the same exposure level. In fact, the same linear two-compartment model as shown in Figure 8 was used to characterize the pharmacokinetics of inhaled styrene in humans. The steady state blood concentration was approximately 0.9 μg per ml, and the terminal half life value was approximately 13 hours. The equivalence of the steady state blood concentrations at the same exposure level, and the first order kinetic characteristics in rats and humans are typical of the pharmacokinetic similarities at nonsaturating dose levels that lend confidence to the extrapolation of data obtained at lower exposure levels in experimental animals to that expected in humans.

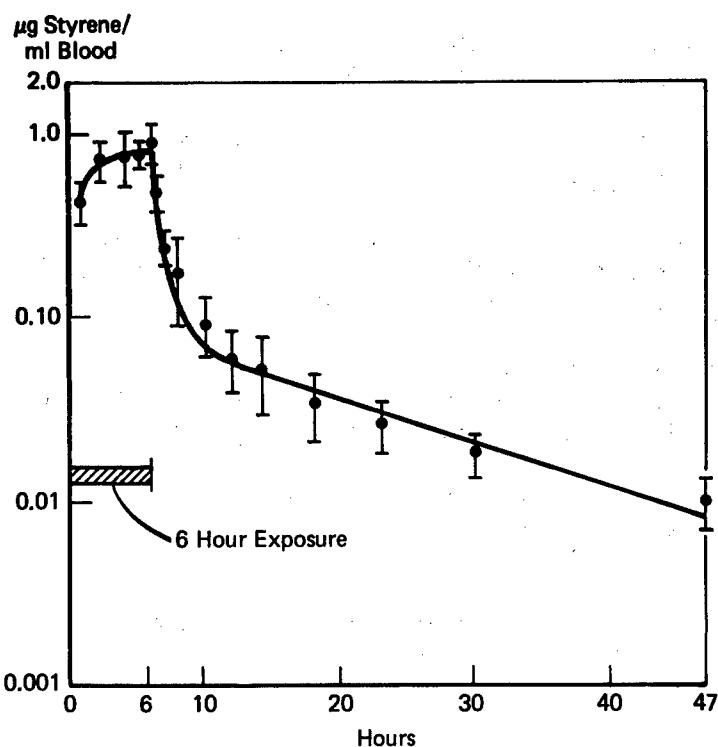


Figure 10. Concentration of styrene in blood of 4 human volunteers exposed to 80 ppm for 6 hours. Data points are mean \pm standard deviation. Solid line is predicted concentration calculated with the best pharmacokinetic parameter estimates of the model shown in Figure 8.

SUMMARY

In conclusion, the major impact of a nonlinear pharmacokinetic profile upon the extrapolation of toxicity data is that at dose levels sufficiently high to elicit nonlinear kinetic characteristics, the relationship of the internal concentration of the toxic entity to the applied dose level (or exposure level) is not representative of that maintained at nonsaturating (linear) dose levels. Therefore, it is essential that toxicity data be considered in conjunction with the pharmacokinetic profile over a sufficient range of dose levels to determine within what dose range a transition from linear to nonlinear kinetics may occur.

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**A PHYSIOLOGICALLY-BASED PHARMACOKINETIC MODEL FOR
2,5-HEXANEDIONE***

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INTRODUCTION

The study of pharmacokinetic events that occur in the body following its exposure to foreign chemicals has been one area in which biomathematical modeling has seen many applications. Most notable to date have been the pharmacologic efforts in which the monitoring of drug levels is of primary concern, particularly in cancer chemotherapy (Bischoff et al., 1971; Dedrick et al., 1973a; Dedrick et al., 1973b). Pharmacokinetic models have also been used in toxicologic studies to investigate the rates of absorption and elimination of environmental contaminants. However, the use of physiologically-based models as an aid in determining the concentration of toxic substances at a relevant site of action has not been extensive, although the benefits of such analyses have been recognized (Bischoff, 1977; Bungay et al., 1978; Dedrick, 1981). A major advantage of the physiologically-based concept is that the compartments in these models correspond to actual regions so that concentration behavior in individual tissues and the incorporation of specific biochemical events in each organ can be achieved. In addition, predictions outside the range of experimental data are generally reliable since the model framework is constructed according to actual physiologic mechanisms. This is in contrast to the so-called "classical models" in which compartments are chosen so as to describe available plasma concentration information. One decides on the complexity of these models based on the number of exponential terms which are needed to best fit the data following a

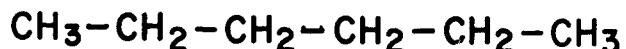
* Nomenclature for this paper can be found in the Appendix.

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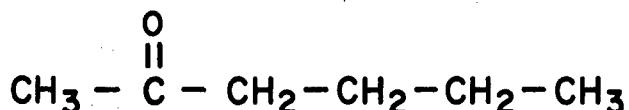
bolus input. Relevant parameters are then evaluated via statistical routines making this approach empirically-based and not amenable to extrapolation to different doses or from one animal species to another, features which are afforded by physiologic models.

Recent reviews by Himmelstein and Lutz (1979) and Chen and Gross (1979) trace the history of physiologically-based pharmacokinetic modeling, and the methodology has been comprehensively discussed by Lutz et al. (1980). The toxicologic applications have primarily addressed the pharmacokinetics of the halogenated hydrocarbons dieldrin (Garrettson and Curley, 1969; Lindstrom et al., 1974), polybrominated biphenyls (Tuey and Matthews, 1980a), polychlorinated biphenyls (Lutz et al., 1977; Tuey and Matthews, 1980b; Bungay et al., 1979), and kepone (Bungay et al., 1979).

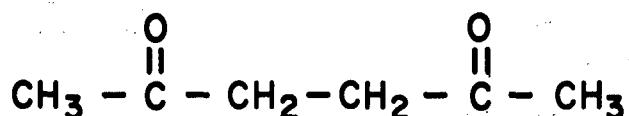
This study examined the pharmacokinetic behavior of 2,5-hexanedione (2,5-HD), the neurotoxic metabolite of the industrial solvents n-hexane and methyl n-butyl ketone (MnBK), a group of compounds which have become known as the neurotoxic hexacarbons (figure 1). The principal objective was to gather relevant tissue distribution and excretion data from rats that had been exposed to ^{14}C -labeled 2,5-HD, and to use this information in constructing a model which would predict the concentration history of radiolabeled material in relevant body compartments, including the nervous system, following a long-term dosing regimen at a dose level of realistic magnitude.



n - HEXANE



METHYL n-BUTYL KETONE



2, 5 - HEXANEDIONE

Figure 1. The neurotoxic hexacarbons, n-hexane and methyl n-butyl ketone, are common organic solvents. 2,5-Hexanedione is an oxidation product that results from the in vivo metabolism of the solvents.

2,5-HEXANEDIONE TOXICITY

The neurotoxic properties of certain hexacarbon solvents have been receiving much attention in toxicology research since an outbreak of neuropathy occurred in a fabrics plant in 1973 (Billmaier et al., 1974). The solvent, methyl n-butyl ketone, was implicated as the agent responsible for the toxicity, and since then exposure to another hexacarbon solvent, n-hexane, has been shown to produce the same types of neurotoxic symptoms. Recent reviews (Spencer et al, 1980; Spencer and Schaumburg, 1980) detail the history of the experimental investigations and give an overview of the biochemical and neurologic phenomena involved with hexacarbon neuropathy.

The causative agent has been shown to be the diketone 2,5-hexanedione, a product of the in vivo metabolism of absorbed n-hexane and MnBK. Figure 2 shows a biochemical reaction pathway that traces the metabolic history of the hexacarbon solvents through the various biotransformation and elimination reactions believed to occur in the body.

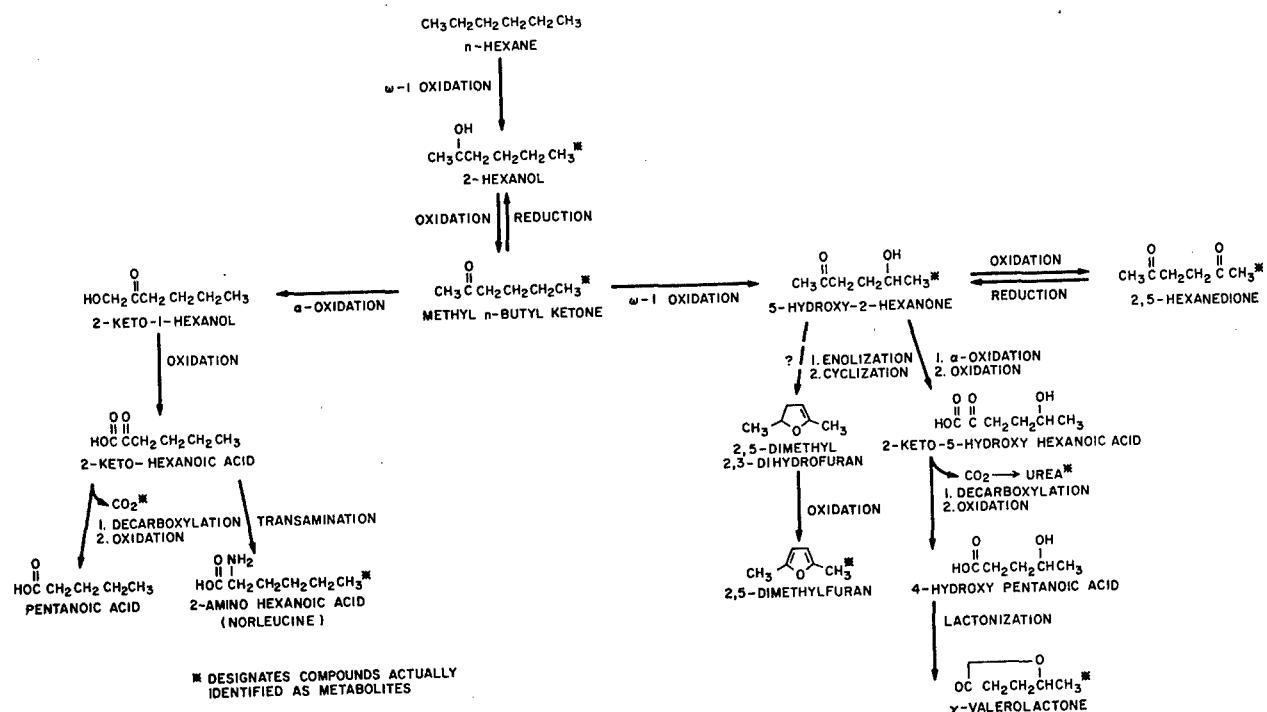


Figure 2. Proposed pathway for the in vivo metabolism of the neurotoxic hexacarbons. This reaction network combines the work of DiVincenzo and co-workers (1977) and Couri and co-workers (1978).

It has been shown that the presence of 2,5-hexanedione at the principal sites of toxicity, the sciatic nerves, can cause the axonal damage which characterizes the neuropathologic condition known as central-peripheral distal axonopathy. The loss of the motor and sensory ability of the hindlimbs, termed hind-limb foot drop, is the result of 2,5-hexanedione exerting its toxic effect directly on the axons of the sciatic nerves.

A host of biochemical theories exist which attempt to explain the nature of the toxicity (Sabri et al., 1979a; Sabri et al., 1979b; Graham and Abou-Donia, 1980; Gillies et al., 1980; Gillies et al., 1981a; Gillies et al., 1981b), but it is generally accepted that 2,5-hexanedione, acting directly on the nerve (probably at the nodes of Ranvier), is first in a line of events which lead to the abnormal pathology.

MATERIALS AND METHODS

A detailed description of the experimental procedures has been reported (Angelo, 1981). Therefore, only a brief summary follows.

SINGLE BOLUS DOSE STUDIES

An investigation of radiolabeled distribution and excretion following single dose exposures of ^{14}C -2,5-hexanedione was accomplished through administration of a radioactive solution of 2,5-HD via ip injection into the male, Sprague-Dawley rats that averaged 250 grams in weight. Unlabeled 2,5-hexanedione (purity > 98%, Eastman Organic Chemicals, Rochester, New York) was combined with $[1,6-^{14}\text{C}]$ 2,5-hexanedione having a specific activity of 2mCi/mmmole (New England Nuclear, Boston, Massachusetts) to achieve dose levels of 8.0 mg/kg and 0.8 mg/kg. These doses were selected so that tissue concentrations of radiolabel were similar to those achieved following inhalation exposure to volatile hydrocarbon solvents at realistic exposure levels (Angelo, 1981).

Metabolism cages provided means for separate collection of urine and feces during exposure. Gas-scrubbing columns containing a solution of mono-ethanolamine and ethylene glycol monomethyl ether (1:2, v/v) were used to collect expired $^{14}\text{CO}_2$. Food and water were provided ad libitum.

Animals were sacrificed by decapitation at selected times ranging from 15 minutes to 72 hours post-exposure. Tissues removed for analysis included liver, kidney, skeletal muscle, lumbar-dorsal fat, skin, lung, sciatic nerve, and spinal cord. Whole blood was collected and separated into plasma and hematocrit fractions via centrifugation. Specimens were frozen until ^{14}C -content was quantified via liquid scintillation counting following tissue oxidation.

Since total radioactivity does not differentiate between parent ^{14}C -2,5-HD and its radiolabeled metabolites, a method was devised to characterize radioactivity in selected tissues using high pressure liquid chromatography. Tissue samples were prepared according to the perchloric acid extraction procedure of Hauschka (1973) and, following neutralization, the acid soluble fraction (ASF) was injected onto the HPLC column. Constant volume fractions were collected from the column effluent, and radioactivity was associated with the 2,5-HD peak appearing on the UV chromatogram. Non-parent radiolabel was not identified structurally, but tissue extraction did yield information on the nature of metabolites as being either soluble or insoluble in the acidic (aqueous) extraction phase.

At times up to 8 hours following a single ip injection of an 8.0 mg/kg dose of ^{14}C -2,5-HD (which was undiluted with cold 2,5-HD), selected tissues were removed and the relative amounts of parent compound and labeled metabolites were determined.

MULTIPLE BOLUS DOSE STUDIES

A group of six male Sprague-Dawley rats, each weighing about 250 grams, received daily ip injections of $[1,6-^{14}\text{C}]$ 2,5-hexanedione in normal saline at a dose level of 8.0 mg/kg. The rats were housed in individual cages and received water ad libitum, but each animal was limited to 15 gm/day of food to keep its weight fairly constant.

The rats received injections every 24 hours for eight consecutive days. Following the final dose, two rats were sacrificed and blood, plasma, and tissues were collected and frozen. Two additional rats were sacrificed 24 hours following the final exposure, that is, at the time when the next injection would have been administered. Specimens were again collected and frozen. The remaining pair of animals was maintained on the weight control diet for 21 days following the eighth injection. This allowed for an analysis of the rate of tissue turnover by examining the decrease in the level of incorporated radiolabel over an extended period of time. Samples from the latter animals were collected and frozen after the three weeks had expired.

CONSTANT INTRAVENOUS INFUSION

Shen and Gibaldi (1974) noted that a constant-rate intravenous infusion achieved the proper condition for estimating more reliable distribution ratios than those obtained from transient data following a bolus injection. Therefore, a surgically implanted Alzet Osmotic Minipump (Alza Corporation, Palo Alto, California)

provided for the controlled release of ^{14}C -2,5-HD solution into the bloodstream of rats while avoiding metabolic complications introduced by prolonged anesthesia.

The surgical procedures have been described previously (Angelo, 1981). After operation, an animal was placed in a cage and received a restricted diet of 15 gm/day of food although water was provided ad libitum. Four rats served as subjects of a constant infusion, continued for 113 hours (~4 1/2 days) in two rats, and the other pair was exposed for 72 hours (3 days). A steady-state condition was verified by blood analysis and $^{14}\text{CO}_2$ production during the course of the infusion.

At the time of necropsy, rats were anesthetized with ether, and the sutures were opened to remove the pump and catheter. The animals were decapitated and blood, plasma, and tissues were collected and frozen.

Oxidation of tissue homogenates prepared the samples for ^{14}C -analysis by liquid scintillation counting, and extraction and HPLC separation was performed on selected tissues to characterize the radioactivity as to ^{14}C -2,5-HD, soluble ^{14}C -metabolites, or incorporated radiolabel.

MODEL DEVELOPMENT

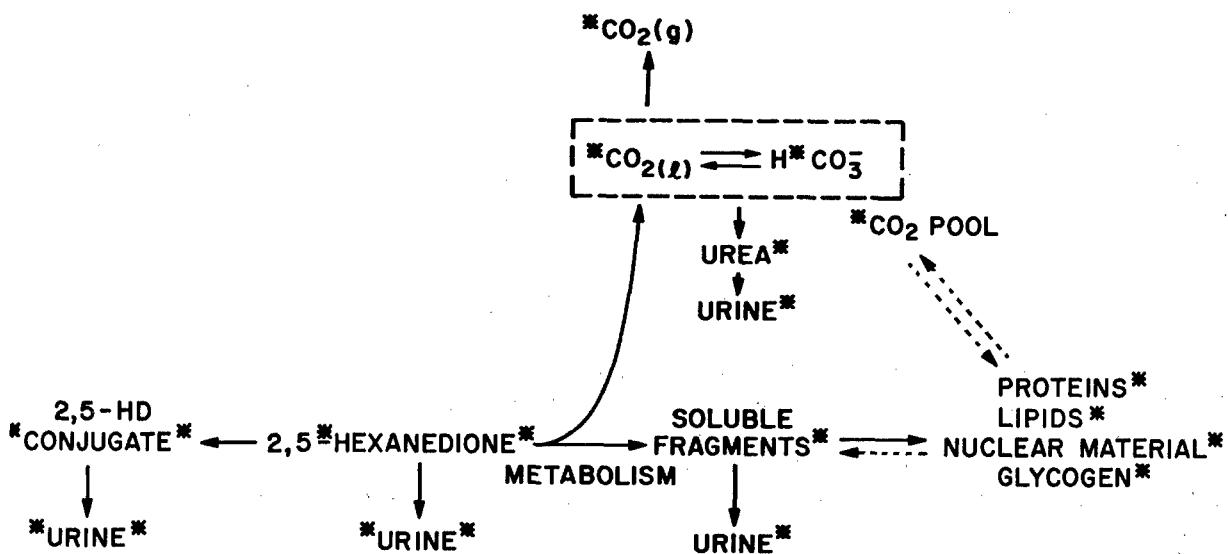
The tissue distribution of radioactivity in rats dosed with ^{14}C -2,5-HD in the single and multiple bolus dose studies and in the constant infusion exposure revealed widespread occurrence of the radiolabel. Following the single 8.0 mg/kg dose, the radioactivity in plasma, liver, kidney, muscle, mixed brain, and lung appeared as parent compound and as acid-soluble or insoluble radiolabeled metabolites, the latter being the ^{14}C which had become covalently bound to undetermined cellular constituents. Additionally, the radioactivity persisted in all tissues at measurable levels up to 72 hours following the single dose exposures.

Respiratory $^{14}\text{CO}_2$ was rapidly produced following the single ip doses of 8.0 mg/kg and 0.8 mg/kg indicating that metabolism of ^{14}C -2,5-HD was a primary elimination route. Most of the radioactivity was respired in the initial 8 hours following dose administration, and about 45% of the dose was accounted for at 72 hours. Similarly, cumulative excretion of radiolabel approached 45% of the dose at 72 hours following the single ip administrations.

LUMPED REACTION SCHEME FOR 2,5-HEXANEDIONE METABOLISM

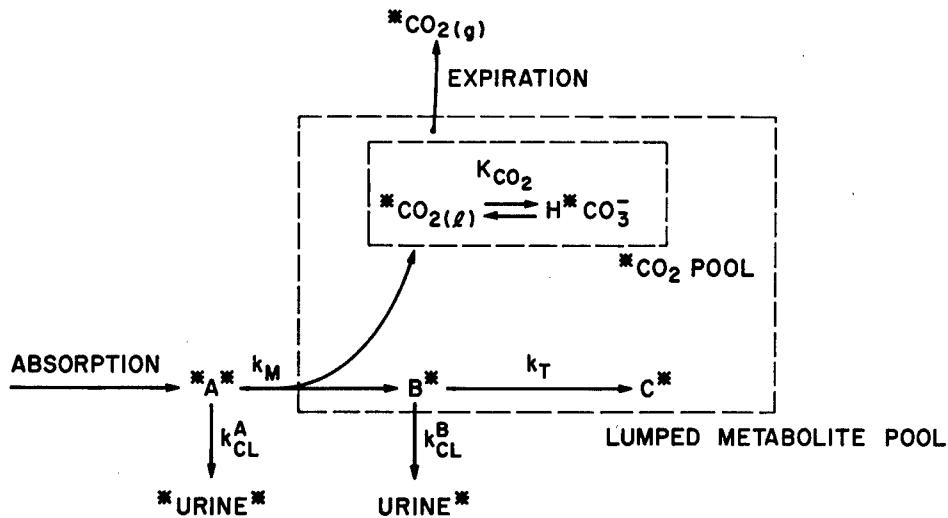
Based on the experimental evidence, a simplified kinetic scheme which described the metabolic history of radiolabel from parent ^{14}C -2,5-HD to metabolites and excretion products was

formulated as depicted in figure 3. Figure 4 illustrates a less complicated version without the minor metabolic pathways and with the radiolabeled metabolites designated as "B" and "C" corresponding to pools of circulating and bound ^{14}C , respectively. Parent ^{14}C -2,5-HD is symbolized as "A". This representation is not a one-compartment description of the body, but rather a history of 2,5-HD catabolism with kinetic events occurring in different body regions. The rate constant for the B+C reaction is tissue specific and is related to the level of metabolic activity of the tissue, i.e. tissue turnover. This scheme along with its kinetic implications has been described previously (Dedrick, 1979; Gillette, 1977; Gehring and Blau, 1977), and was used here in conjunction with a whole-body model to describe the radiolabeled species and subsequent reactions.



* DENOTES PRESENCE OF RADIOLABEL

Figure 3. Hypothetical pathway showing the involvement of 2,5-hexanedione and its associated radiolabel in intermediary metabolism. The dashed arrows represent less prominent pathways for involvement of radiolabel.



* DENOTES PRESENCE OF RADIOLABEL

Figure 4. Symbolic representation of 2,5-hexanedione metabolism. A, B, and C are lumped parameters referring to the parent compound (2,5-HD), soluble metabolites and covalently-bound (incorporated) metabolites, respectively.

MODEL FRAMEWORK

The physiologic model describing the pharmacokinetics of 2,5-hexanedione is illustrated in figure 5. The compartments depicted in the diagram correspond to actual anatomical regions in the rat body, and the total volume represented by the sum of individual compartment sizes for > 87% of the total body mass. Transport of parent compound and soluble metabolites throughout the system is governed by individual plasma flow rates to different regions. The mass transfer of administered 2,5-HD via injection into the hepato-portal system is shown schematically by a double arrow. Compartments are included to account for important physiologic events such as metabolism in the liver and clearance by the kidneys. A lung compartment is included to account for future extensions of the model that describe respiratory uptake and excretion of volatile toxins. A lean compartment is a necessary addition since it affects the distribution of materials due to the large percentage of body mass it represents. The peripheral and central nervous systems are important compartments since they are the sites of the toxic lesions induced by exposure to 2,5-hexanedione. Anatomy such as the heart, spleen, skeletal system, and smaller organs have been excluded since they exert little influence on the distribution patterns of parent compound or metabolites.

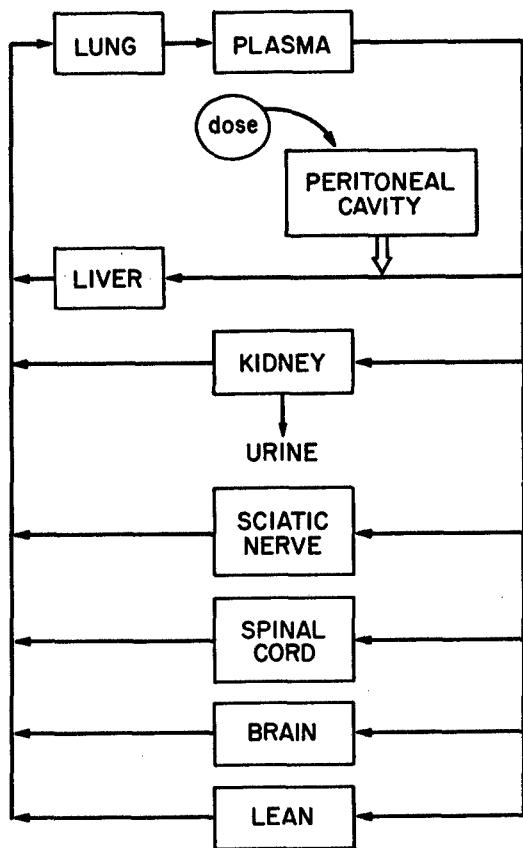


Figure 5. Schematic flow diagram of important body compartments in a physiologically-based model for 2,5-hexanedione. Single arrows represent plasma flows, and the double arrow indicates mass transfer from the peritoneal cavity to the hepato-portal system.

Tissues having a low blood perfusion have been lumped into a "lean" compartment. This is the largest region in the body and includes the muscle, skin, and fat tissues. Muscle and skin have roughly the same perfusion which is substantially lower than the blood flow to the viscera. Fat tissue, although it is not lean *per se*, behaves similarly to the muscle and skin in that no large "storage" effect is seen with 2,5-HD or its metabolites. Some environmental toxins such as polychlorinated biphenyls (PCB's) have been shown to reside for a long period of time in adipose tissue. This is also true for highly lipid soluble drugs such as thiopental (Bischoff and Dedrick, 1968). The depot behavior of chemicals is reflected pharmacokinetically by large values of fat tissue/plasma (or fat tissue/blood) distribution ratios. The behavior of rat tissue toward 2,5-HD was such that the distribution ratio was of the order one, whereas PCB's have ratios ranging from

30 to 400. In light of this, the fat region caused no extraordinary distribution patterns to arise with respect to 2,5-HD, so model simplification was achieved by lumping the three tissues into one compartment.

Fecal samples were analyzed in a number of rats exposed to single ip injections of ^{14}C -2,5-HD, and data indicated that very little radiolabel left the body by this route. Not more than 3% of the total dose was eliminated up to 72 hours postexposure, a finding consistent with the observations of DiVincenzo et al. (1977) who collected less than 2% of the radioactive dose in fecal material following oral exposures to [$1-^{14}\text{C}$] MnBK. Therefore, a GI compartment was not included in the preliminary model since biliary secretion of radiolabel, if it did occur, was followed by complete reabsorption in the gut.

A simple flow-limited model was used as a first assumption to describe the diffusion equilibrium that existed between a compound in the vascular and extravascular spaces of tissues. Basically, the approximation implies that membrane permeabilities are so much larger than the regional blood perfusion that the diffusion equilibrium becomes strictly dependent upon the amount of chemical supplied by the arterial flow.

Since the extent of binding of 2,5-HD to the plasma protein albumin is less than 10% (Angelo, 1981), linear binding was used in the model equations. First-order terms were used for all rate processes except for the spinal cord compartment where a nonlinear rate expression was used to describe the B+C reaction. Except for the plasma compartment where it was necessary to consider the release of radioactivity from C, the decay in tissue concentrations of incorporated radiolabel was assumed negligible over the 72-hour time scale of model simulations corresponding to single ip administrations.

A system of compartment mass balances (see Appendix) was derived in the form of differential equations that were solved numerically using Gear's method (1971). This produced computer-generated model predictions with specified parameter values and a given dosing schedule.

DETERMINATION OF MODEL PARAMETERS

Table 1 lists the compartments in the model together with their sizes and plasma perfusion rates. Table 2 summarizes the distribution and kinetic parameters that were evaluated for 2,5-HD and metabolites as discussed below.

TABLE 1. COMPARTMENT SIZES AND PLASMA PERFUSION RATES FOR A 250 GRAM RAT

Compartment	Volume ml	Plasma Flow Rate ml/hr
Plasma	13.5 ^{a1}	---
Liver	10.0 ¹	576 ¹
Kidney	2.3 ²	360 ²
Lung	2.9 ³	b
Lean	185 ^c	470 ^d
Brain	3.9 ³	57.6 ^{e4}
Sciatic Nerve	---	4.0 ^f
Spinal Cord	---	10.1 ^g
Peritoneal Cavity	16 ^h	---

¹ Data taken from Lutz et al., 1977.

² Data taken from Bischoff et al., 1971.

³ Data taken from Adolf, 1949.

⁴ Data taken from Mandel et al., 1963.

^aCorrected for 40% hematocrit. ^b100% of cardiac output, or sum of venous flow from each compartment. ^cSum of muscle: 125 ml; skin: 40 ml; and fat: ~20 ml/hr (Lutz et al., 1977). ^dSum of muscle: 270 ml/hr; skin: 180 ml/hr; and fat: ~20 ml/hr (Lutz et al., 1977). ^eCalculated from a blood perfusion rate in whole mixed brain of 24.6 ml/hr-gm. ^fPerfusion rate in ml plasma/hr-gm. ^gPlasma perfusion rate of whole mixed cord. ^hEstimated (Angelo, 1981).

TABLE 2. DISTRIBUTION PARAMETERS

Compartment	Tissue/Plasma Distribution Ratios for 2,5-HD (R_i^A)	Tissue/Plasma Distribution Ratios for Metabolites (R_i^B)	Tissue/Plasma Turnover Ratio (T_i)
Liver	1.7	3.4	0.7
Kidney	0.6	3.1	0.75
Lung	0.6	2.5	0.55
Lean	0.4	0.8	1.0
Brain	0.4	0.5	1.4
Sciatic Nerve	0.4	0.4	3.0
Spinal Cord	0.4	0.5	5.0 ($K_{SC} = 11.5$)

Kinetic Parameters

Peritoneal Permeability - Area Product (PA, ml/hr) 12.0

Kidney Clearance, 2,5-HD (k_{cl}^A , ml/hr) 10.0

Kidney Clearance, Metabolites (k_{cl}^B , ml/hr) 30.0

Metabolism Constant, 2,5-HD (k_{met} , ml/hr) 150.0

^{14}C -Incorporation Rate Constant for Plasma (k_p , hr^{-1}) 0.03

Tissue/Plasma Distribution Ratio (R)

The 2,5-HD tissue/plasma distribution parameters for liver, kidney, muscle, lung, and whole brain were calculated as the ratios of the respective concentrations during steady-state conditions following the constant iv exposure. Values for the spinal cord and sciatic nerve were initially assumed to be similar to whole brain since insufficient tissue mass prevented the use of the chromatographic assay. Subsequently, minor adjustments were made to the initial parameter estimates in order to achieve better agreement between the data and model predictions. Distribution ratios for metabolites were also adjusted slightly following preliminary simulations to obtain a better prediction of data as judged by visual inspection.

Metabolic Rate Constant (k_{MET})

The metabolism rate for each exposure regimen was modeled by a first-order clearance term and was calculated from iv infusion data according to

$$k_{MET} = \frac{^{14}\text{CO}_2 \text{ produced/hr}}{(\text{C}_{2,5-\text{HD}}) \text{ liver, steady-state}} \quad (1)$$

The single dose exposures of 8.0 mg/kg and 0.8 mg/kg produced similar $^{14}\text{CO}_2$ elimination rates, thereby suggesting that 2,5-HD metabolism was not saturated at these dose levels. Additionally, since the liver tissue concentrations achieved in the iv infusions were below the levels of those obtained after the low ip dose, the metabolism kinetics remained in the linear range.

Kidney Clearances (k_{cl})

The kidney clearance of 2,5-HD was estimated from the data of DiVincenzo et al. (1977) by dividing the cumulative amount of 2,5-HD in the urine by the area under the curve for 2,5-HD plasma concentration vs. time. Kidney clearance of ^{14}C -metabolites was approximated by first calculating the overall kidney clearance of radioactive material according to the method just described, and subtracting from this the contribution of ^{14}C -2,5-HD kidney clearance.

Peritoneal Permeability - Area Product (PA)

Data on the peritoneal permeability - area product of water-soluble compounds in rats were provided by Dr. Robert Dedrick of the National Institutes of Health and were used for initial

estimates of PA. The dose was modeled as transferring from the peritoneal cavity directly into the liver since evidence (Lukas et al., 1971) indicated that the major percentage of an ip dose is absorbed in this manner.

Tissue/Plasma ^{14}C -Turnover Ratios (T)

The metabolism of ^{14}C -2,5-HD resulted in a significant amount of radiolabel becoming incorporated into molecular components of rat tissues. The radiolabel, once incorporated, became part of the metabolic turnover cycle, and the level of incorporated ^{14}C decayed with time, reflecting that radioactive components were diluted with unlabeled material. A first-order expression was previously used by Broda (1960) in describing the synthesis and degradation of biomolecules and was applied here in the description of the uptake and release of ^{14}C from tissue components. The initial estimates of the first-order decay constants for specific tissues were obtained according to

$$k_D = \frac{\ln \left(\frac{C_0}{C} \right)}{t} \quad (2)$$

where C_0 and C are the concentrations of incorporated radiolabel in a tissue at time zero and t , respectively. Time zero was chosen to be 24 hours following the final ip dose of ^{14}C -2,5-HD in the multiple dosing schedule. At that point, parent ^{14}C -2,5-HD was no longer present, and virtually all of the radiolabel was in the incorporated form. Twenty-one days later, tissue levels of ^{14}C had been sufficiently diluted by turnover to allow for the calculation of the tissue-specific decay constants according to equation 2.

The tissue/plasma turnover ratios are the quotients of the individual tissue/plasma decay constants, and provide a relative measure of the ^{14}C -releasing ability of tissues as compared to plasma constituents.

To account for the tissue uptake of ^{14}C as described by the $\text{B} \rightarrow \text{C}$ reaction in figure 4, the tissue/plasma turnover ratios were multiplied by the first-order rate constant for ^{14}C -incorporation into plasma proteins, k_p , as documented by Hevesy (1948). The resulting value provided an estimate of the ^{14}C -incorporation rate constants for tissues.

In all tissue compartments, a linear expression for the turnover ratio adequately represented the data except for the spinal cord where a concentration-dependent expression was required to describe the uptake of radiolabel. Accordingly, equation 3 was used:

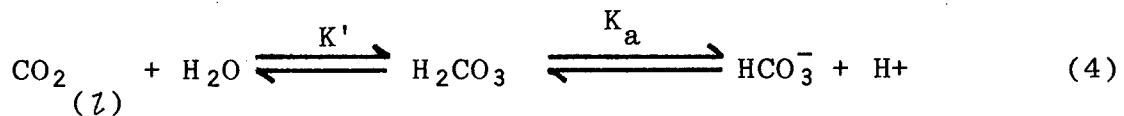
$$T'_{SC} = T_{SC} \left\{ \frac{1}{1 + \frac{C_{SC}^B}{K_{SC}}} \right\} \quad (3)$$

where T'_{SC} and T_{SC} are the apparent and true spinal cord/plasma turnover ratios, respectively; C_{SC}^B is the concentration of soluble ^{14}C -metabolites in the spinal cord; and K_{SC} is a saturation constant for radiolabel incorporation into the spinal cord.

Contribution of Radiolabeled Bicarbonate to Soluble ^{14}C -Metabolites

A substantial amount of ^{14}C -2,5-hexanedione was metabolized in vivo leading to the excretion of $^{14}\text{CO}_2$ in the exhaled breath of rats. Since the decarboxylation occurred in the liver, a finite lag time existed before the $^{14}\text{CO}_2$ was expired via respiration, and as Guyton (1976) has indicated, the carbon dioxide may participate in a number of side-reactions, most notably its conversion to bicarbonate ion. The effect of including a $^{14}\text{CO}_2$ was notable as long as ^{14}C -2,5-HD was available for decarboxylation. The critical time corresponded to an interval of up to eight hours following ip exposure of the parent compound.

Bicarbonate formation from dissolved CO_2 occurs in the water space of the body, i.e. extracellular and intracellular fluid, via the carbonic acid intermediate according to the equilibrium.



Since the amount of bicarbonate ion is appreciable throughout the body, it was expected that dissolved $^{14}\text{CO}_2$ derived from ^{14}C -2,5-HD metabolism would give rise to isotopically labeled bicarbonate ion. Using the equilibrium expressions and a pseudo steady-state mass balance on $^{14}\text{CO}_2$ species in the water spaces of the body, viz.

$$V_{BW} \left[\frac{^{14}\text{CO}_2}{T} \right] = V_{IC} \left\{ \left[\frac{^{14}\text{CO}_2}{(I)} \right] + \left[\frac{\text{H}^{14}\text{CO}_3^-}{(I)} \right] \right\}_{IC} + V_{EC} \left\{ \left[\frac{^{14}\text{CO}_2}{(E)} \right] + \left[\frac{\text{H}^{14}\text{CO}_3^-}{(E)} \right] \right\}_{EC} \quad (5)$$

where V_{BW} = volume of body water

V_{IC} , V_{EC} = volumes of intracellular water and extracellular water, respectively.

an expression relating radiolabeled bicarbonate ion to total $^{14}\text{CO}_2$ in the water space was derived:

$$\frac{[\text{H}^{14}\text{CO}_3^-]}{[\text{H}^{14}\text{CO}_3^-]} = \frac{\frac{\left\{ \frac{V_{IC}}{V_{BW}} \right\} K'}{10^{pK_a - \text{pH}_{IC}}} + \frac{\left\{ \frac{V_{IC}}{V_{BW}} \right\} K'}{10^{pK_a - \text{pH}_{EC}}}}{T \left\{ 1 + \frac{\left\{ \frac{V_{IC}}{V_{BW}} \right\} K'}{10^{pK_a - \text{pH}_{IC}}} + \frac{\left\{ \frac{V_{EC}}{V_{BW}} \right\} K'}{10^{pK_a - \text{pH}_{EC}}} \right\}} \quad (6)$$

Values for constants in equation 6 were obtained from Guyton (1976), viz.

$$\frac{V_{IC}}{V_{BW}} = 0.6$$

$$\frac{V_{EC}}{V_{BW}} = 0.4$$

$$\text{pH}_{IC} = 7.0$$

$$\text{pH}_{EC} = 7.4$$

The accumulation of $\text{H}^{14}\text{CO}_3^-$ over time was calculated from equation 6 and a mass balance on total $^{14}\text{CO}_2$ accumulation:

$$\left\{ \begin{array}{l} \text{accumulation of } ^{14}\text{CO}_2 \\ \text{in body water} \end{array} \right\} = \left\{ \begin{array}{l} ^{14}\text{CO}_2 \text{ production via} \\ ^{14}\text{C}-2,5-HD \text{ metabolism} \\ \text{in liver} \end{array} \right\} - \left\{ \begin{array}{l} \text{rate of ex-} \\ \text{piration of} \\ ^{14}\text{CO}_2(g) \text{ via} \\ \text{lungs} \end{array} \right\}$$

where the last term was fit to $^{14}\text{CO}_2$ excretion data using an empirical expression. At each integration step in time, the calculated value of $\text{H}^{14}\text{CO}_3^-$ concentration was added to the concentration of ^{14}C -metabolites in each tissue compartment to account for the temporary hold-up of $^{14}\text{CO}_2$ in the water space before it was exhaled. The contribution of dissolved $^{14}\text{CO}_2(g)$ was considered

negligible. In addition, a conversion factor of 0.8 ml H₂O/gram of tissue (Altman and Dittmer, 1972) was applied to H¹⁴CO₃ concentration since it was based on ml of water and output tissue concentrations were in terms of ml of tissue.

MODEL SIMULATIONS AND COMPARISONS WITH DATA

SINGLE DOSE EXPOSURE

Figures 6 through 11 depict experimental tissue distribution data and the corresponding model predictions for single ip doses of 0.8 mg/kg and 8.0 mg/kg of [1,6-¹⁴C] 2,5-hexanedione. These graphs reflect values of total radioactivity concentration in each compartment which is the sum of ¹⁴C-2,5-HD and labeled metabolite concentrations obtained from the solution of the model equations. In general, the predictions are good for plasma, sciatic nerve, liver, kidney, lung, and lean compartments. The model does very well in predicting the data in the elimination phase although the absorption phase data are underpredicted somewhat up to one hour postexposure. Additionally, the pharmacokinetics do not exhibit any dose dependency in these figures. This is not the case, however, for the spinal cord compartment as shown in figure 12 where the disparity between a linear ¹⁴C-incorporation phenomenon and the dose-dependent behavior is illustrated. The dashed line reflects the solution of the model when a linear turnover ratio was assumed to exist between the low and high dose levels.

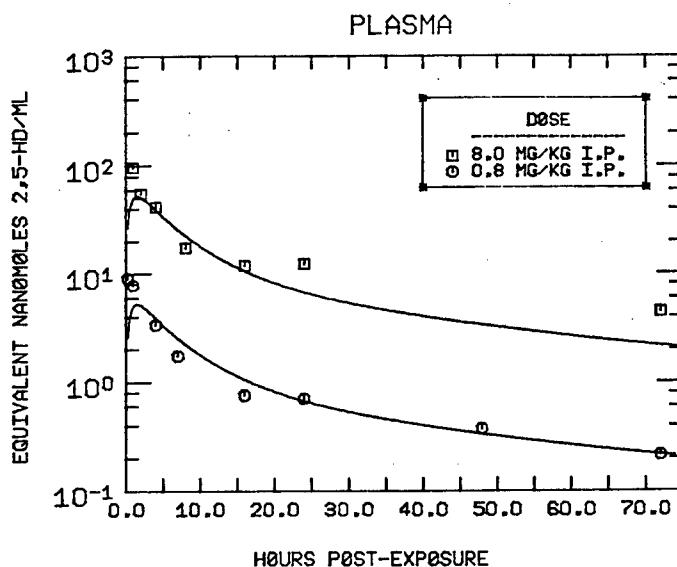


Figure 6. Plasma concentration profiles of total radioactivity following single bolus doses of [1,6-¹⁴C] 2,5-hexanedione in 250 gm rats. The curves represent the model simulations, and each data point is the average concentration value for two or three animals.

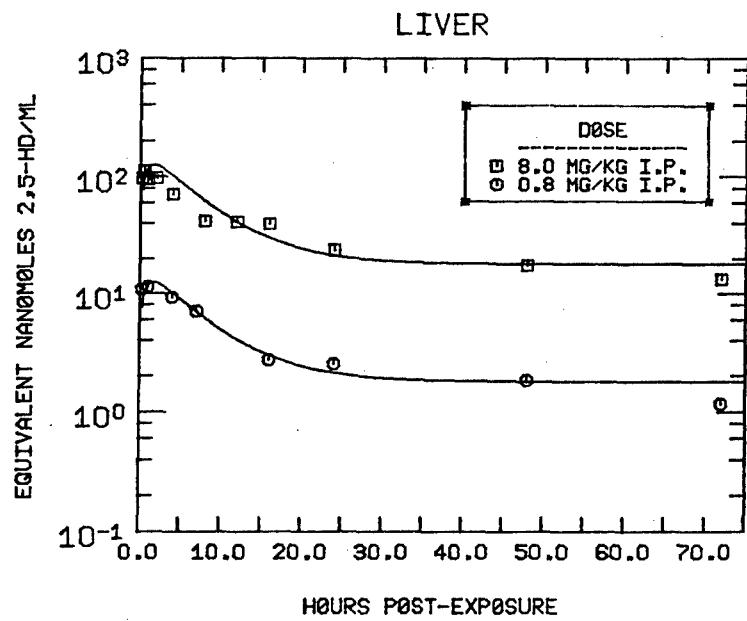


Figure 7. Liver concentration profiles of total radioactivity following single bolus doses of $[1,6-^{14}\text{C}]$ 2,5-hexanedione in 250 gm rats. The curves represent the model simulations, and each data point is the average concentration value for two or three animals.

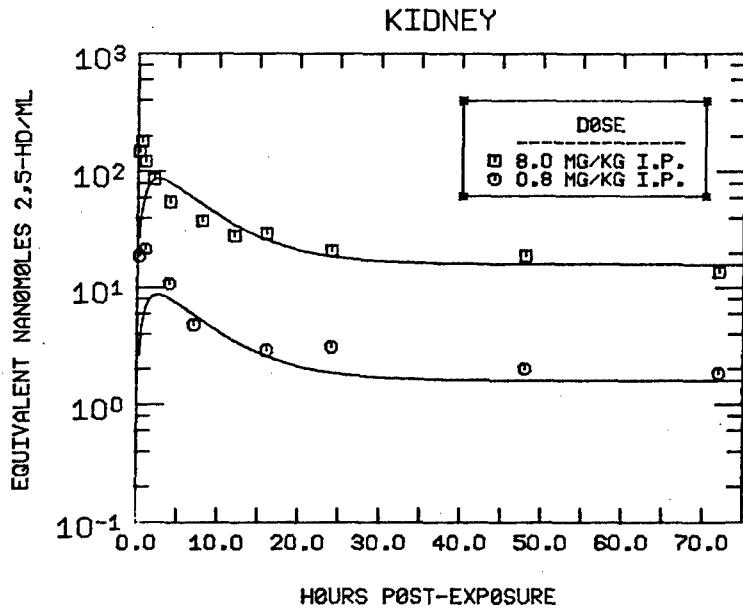


Figure 8. Kidney concentration profiles of total radioactivity following single bolus doses of $[1,6-^{14}\text{C}]$ 2,5-hexanedione in 250 gm rats. The curves represent the model simulations, and each data point is the average concentration value for two or three animals.

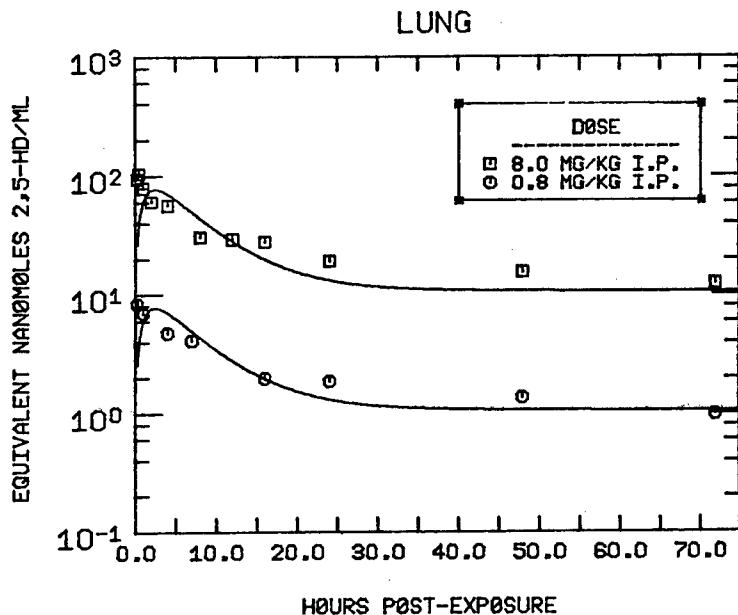


Figure 9. Lung concentration profiles of total radioactivity following single bolus doses of $[1,6-^{14}\text{C}]$ 2,5-hexanedione in 250 gm rats. The curves represent the model simulations, and each data point is the average concentration value for two or three animals.

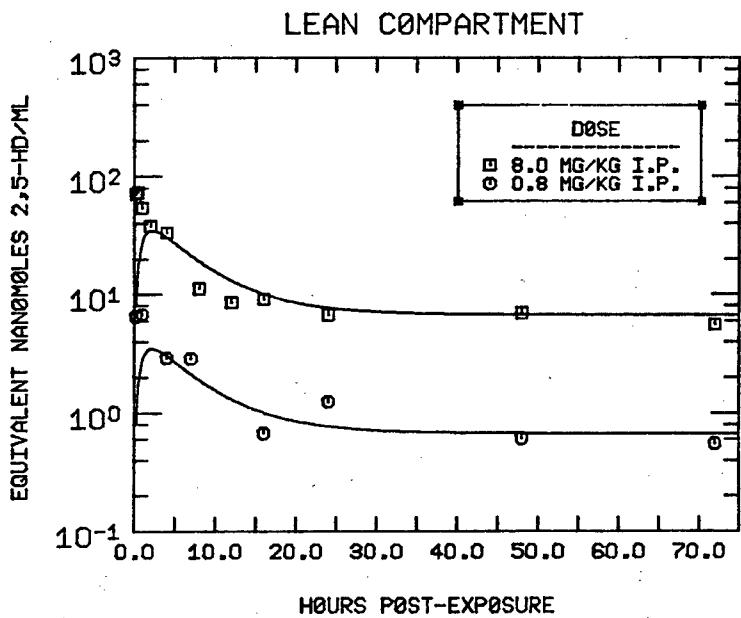


Figure 10. Lean tissue concentration profiles of total radioactivity following single bolus doses of $[1,6-^{14}\text{C}]$ 2,5-hexanedione in 250 gm rats. The curves represent the model simulations, and each data point is the average concentration value for two or three animals.

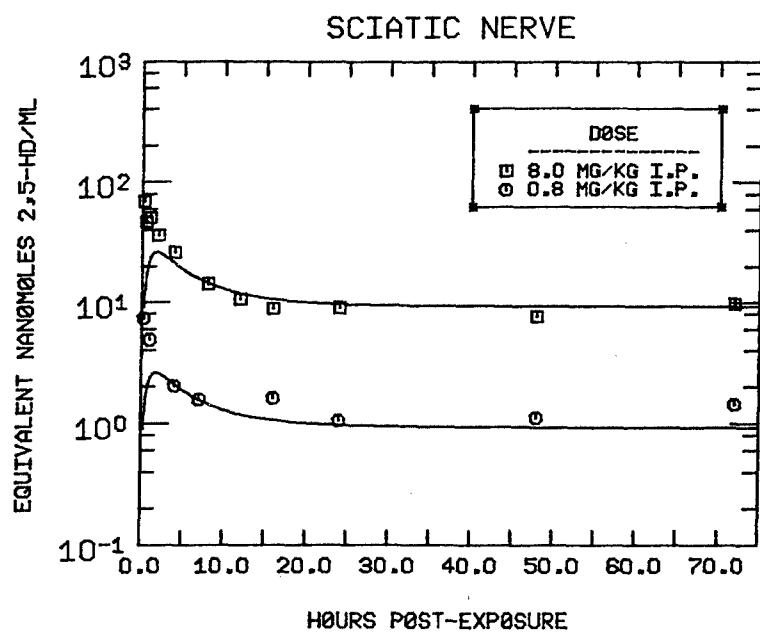


Figure 11. Sciatic nerve concentration profiles of total radioactivity following single bolus doses of $[1,6-^{14}\text{C}]$ 2,5-hexanedione in 250 gm rats. The curves represent the model simulations, and each data point is the average concentration value for two or three animals.

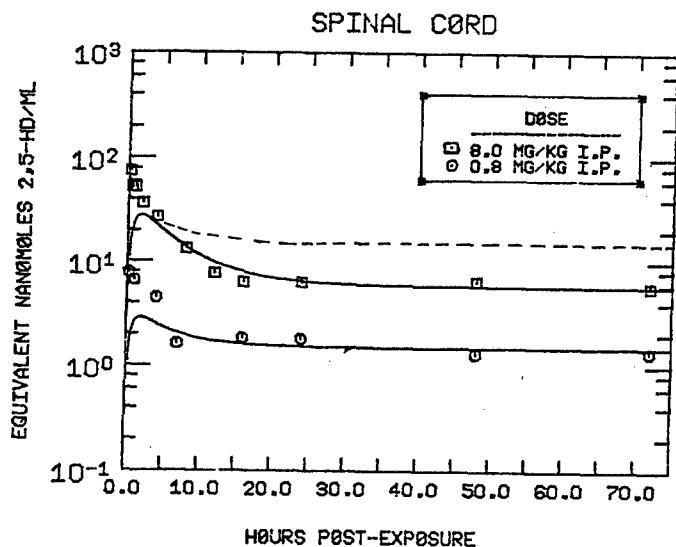


Figure 12. Spinal cord concentration profiles of total radioactivity following single bolus doses of $[1,6-^{14}\text{C}]$ 2,5-hexanedione in 250 gm rats. The solid curves represent the model simulations, and the dashed curve is the simulation result assuming a dose-independent tissue/plasma turnover ratio for the spinal cord. Each data point is the average concentration value for two or three animals.

Figures 13 and 14 show the model predictions for unchanged 2,5-HD, labeled metabolites, and total radioactive species for plasma and liver. The distribution behavior illustrated in these figures is representative of the other tissue compartments examined. The 2,5-HD was eliminated rapidly and was close to or below the limit of detection by 8 hours after exposure. Very good agreement is seen in the model's prediction of the experimental data for the plasma compartment up to 24 hours postexposure. The elimination half-life of 2,5-HD from plasma was calculated from the slope of the terminal portion of the curve to be 2.3 hours. This value is in reasonable agreement with the half-life of 1.7 hours of 2,5-HD in guinea pig serum reported by DiVincenzo et al. (1976). The labeled metabolites and total radioactivity as predicted in figure 13 also agree well with the data.

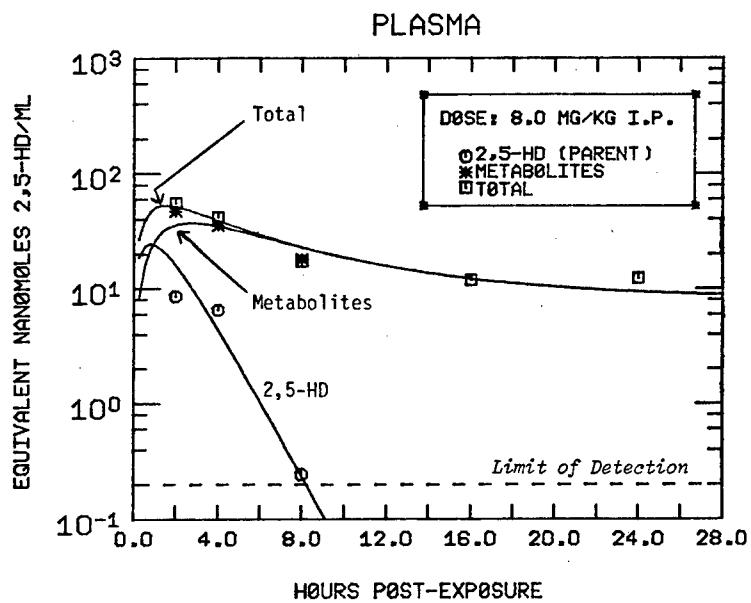


Figure 13. Distribution of radioactivity in plasma as ^{14}C -2,5-hexanedione and labeled metabolites following a single 8.0 mg/kg ip dose of $[1,6-^{14}\text{C}]$ 2,5-hexanedione in rats. The curves represent model simulations, and the data points are from pooled samples of two animals.

The distribution in the liver compartment is represented in figure 14. The data for 2,5-HD indicate that the parent species was detectable at relatively high levels up to 4 hours postexposure followed by rapid elimination between 4-8 hours. The model predicts this behavior to a reasonable extent although the data point at 4 hours is underpredicted by the simulation. The agreement between metabolites and total radiolabel concentration data and model predictions is good up to 24 hours postexposure.

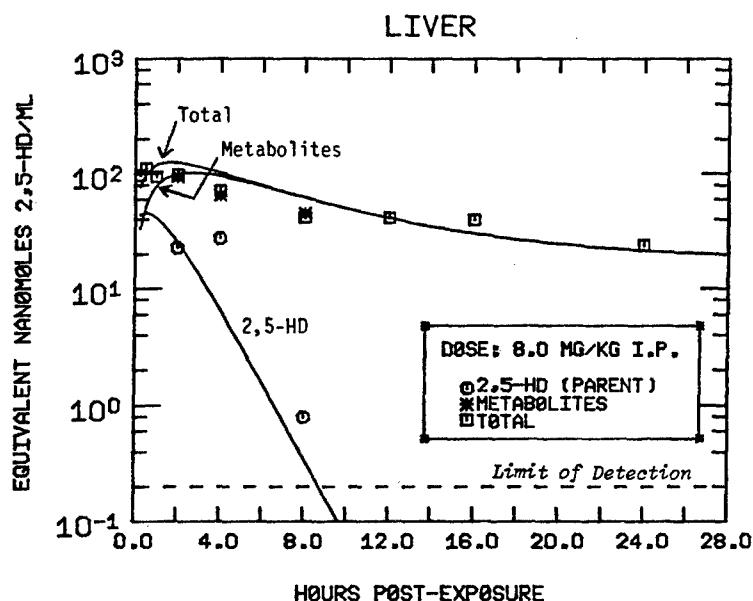


Figure 14. Distribution of radioactivity in liver as ^{14}C -2,5-hexanedione and labeled metabolites following a single 8.0 mg/kg ip dose of $[1,6-^{14}\text{C}]$ 2,5-hexanedione in rats. The curves represent model simulations, and the data points are from pooled tissues of two animals.

The contribution of radioactive bicarbonate, $\text{H}^{14}\text{CO}_3^-$, formed as a result of its equilibrium with $^{14}\text{CO}_2$ produced from the metabolism of ^{14}C -2,5-HD was found to be important in describing the distribution of radioactive metabolites at short times (less than 2 hours postexposure). Including this contribution produced a more quickly rising curve for metabolite concentration in figures 13 and 14. The hold-up of $^{14}\text{CO}_2$ as $\text{H}^{14}\text{CO}_3^-$ (before it is eliminated via respiration) appeared to be an important point to consider when accounting for total radiolabel in tissues.

URINARY EXCRETION

Figure 15 shows the data and model predictions of cumulative urinary ^{14}C excretion, expressed as percent of dose, in rats that received a single 8.0 mg/kg ip dose of $[1,6-^{14}\text{C}]$ 2,5-hexanedione. The data represent total radioactivity found in the urine, and the curve is the sum of parent and metabolite clearances as predicted by the model. Radioactivity was excreted rapidly by this route for about 8 hours, then more slowly up to 24 hours postexposure, after which time the amount excreted became negligible. The

model predicts that about 45% of the total dose would be eliminated by 72 hours following exposure. Also shown is the model prediction for the urinary excretion of unchanged ^{14}C -2,5-HD which amounts to less than 5% of the total dose at 72 hours postexposure.

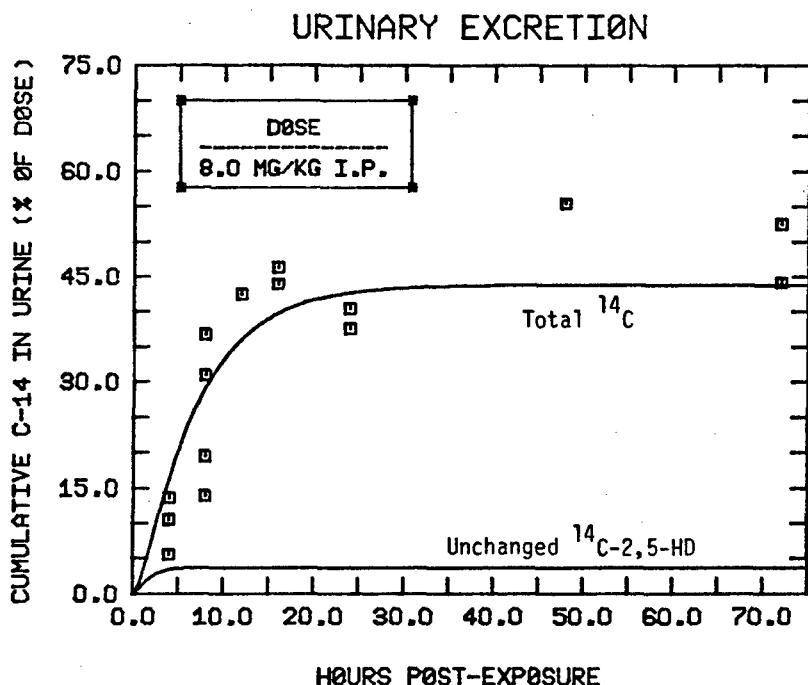


Figure 15. Model predictions of total ^{14}C excreted in the urine following a single 8.0 mg/kg ip dose of [1,6- ^{14}C] 2,5-hexanedione in rats. Also shown is the model prediction of unchanged ^{14}C -2,5-HD that would be eliminated by renal clearance. Data points represent single observations of cumulative ^{14}C collected in urine as percent of dose.

Figure 16 depicts similar urinary excretion data for the case of a 0.8 mg/kg ip dose. As with figure 15, the model predicts about 45% of the total dose being eliminated in the urine after 72 hours, and less than 5% of it as unchanged ^{14}C -2,5-HD.

The observations in figure 15 and 16 are supported by the findings of DiVincenzo et al. (1977) who reported a total urinary elimination of 35.1% of the dose at 48 hours following an oral dose of 20 mg/kg of ^{14}C -methyl n-butyl ketone in rats, and 39.9% of the dose eliminated via the urine after 48 hours following a 200 mg/kg oral dose of the same material. Additionally, analysis of the urine collected from the 200 mg/kg dose revealed that about 5.5% of the radioactivity was due to ^{14}C -2,5-HD. Even though animals were

dosed with ^{14}C -2,5-HD in this study, comparisons with the ^{14}C -MnBK data of DiVincenzo and co-workers provide a basis for evaluating the performance of this model's predictions which do appear to represent the urinary clearance of total radiolabel and unchanged 2,5-HD rather well.

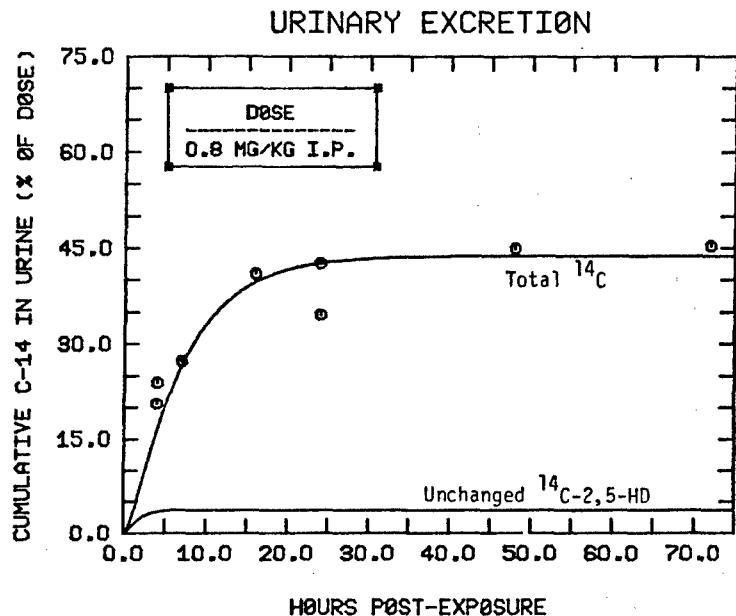


Figure 16. Model prediction of total ^{14}C excreted in the urine following a single 0.8 mg/kg ip dose of $[1,6-^{14}\text{C}]$ 2,5-hexanedione in rats. Also shown is the model prediction of unchanged ^{14}C -2,5-HD that would be eliminated by renal clearance. Data points represent single observations of cumulative ^{14}C collected in urine as percent of dose.

MULTIPLE DOSE EXPOSURES

Figures 17 through 24 are the model predictions for distribution of radiolabel in sciatic nerve, spinal cord, liver, kidney, lean, lung, plasma, and brain following a chronic dosing schedule of daily 8.0 mg/kg ip injections $[1,6-^{14}\text{C}]$ 2,5-hexanedione in rats. The parameter values listed in tables 1 and 2 were used in these simulations, and the data points represent concentration values from animals that received the daily administrations for a total of eight days. These data were collected to provide a basis for verifying that the model could predict tissue distribution behavior following a chronic dosing regimen.

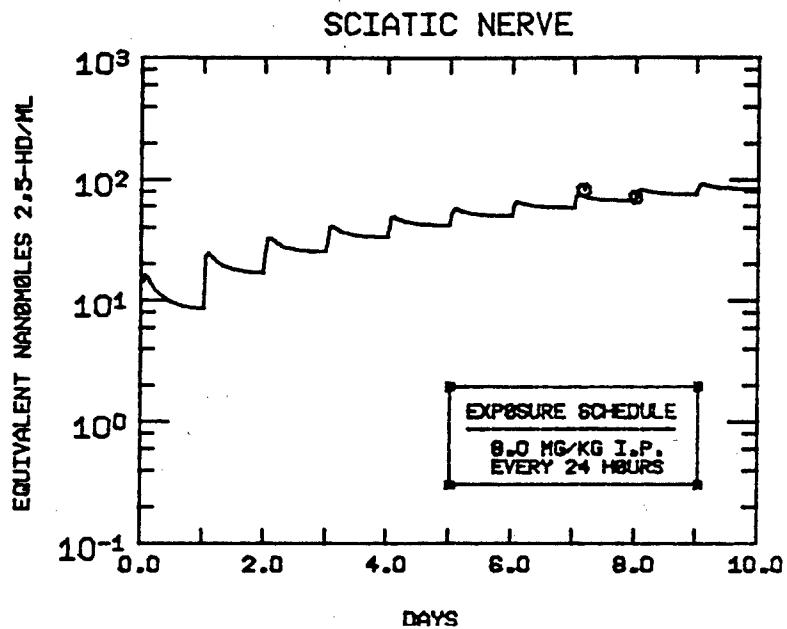


Figure 17. Model prediction of total radiolabel concentration in sciatic nerve tissue of rats following daily ip doses (8.0 mg/kg) of $[1,6-^{14}\text{C}]$ 2,5-hexanedione. Each data point represents pooled samples from two animals.

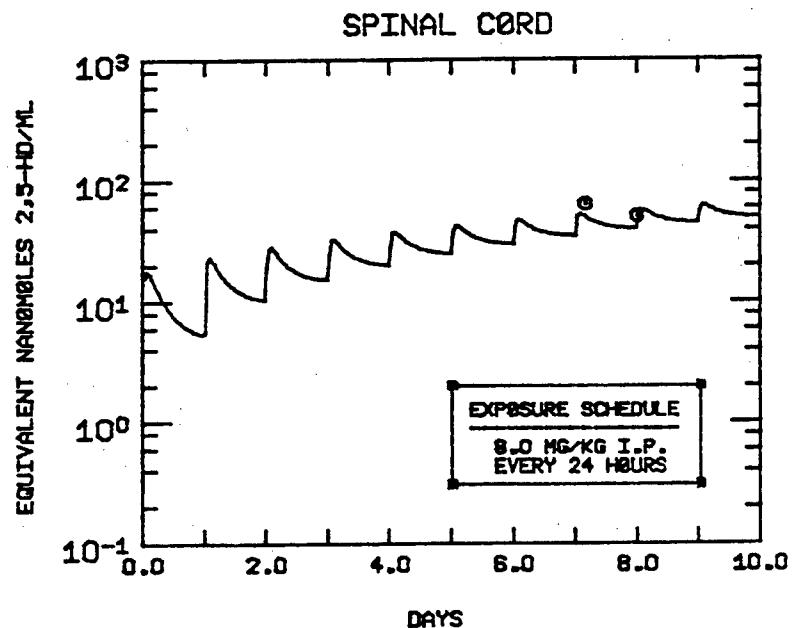


Figure 18. Model prediction of total radiolabel concentration in mixed spinal cord of rats following daily ip doses (8.0 mg/kg) of $[1,6-^{14}\text{C}]$ 2,5-hexanedione. Each data point represents pooled samples from two animals.

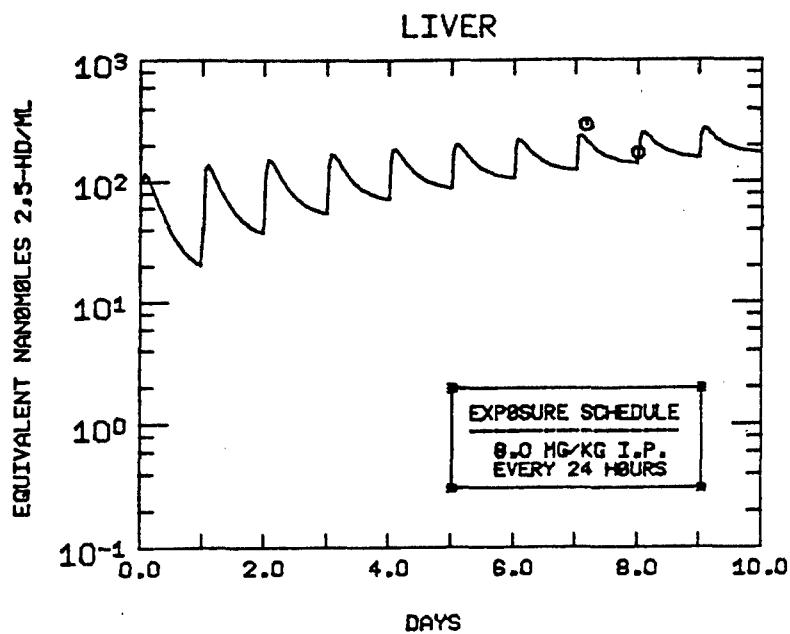


Figure 19. Model prediction of total radiolabel concentration in liver tissue of rats following daily ip doses (8.0 mg/kg) of $[1,6-^{14}\text{C}]$ 2,5-hexanedione. Each data point represents pooled samples from two animals.

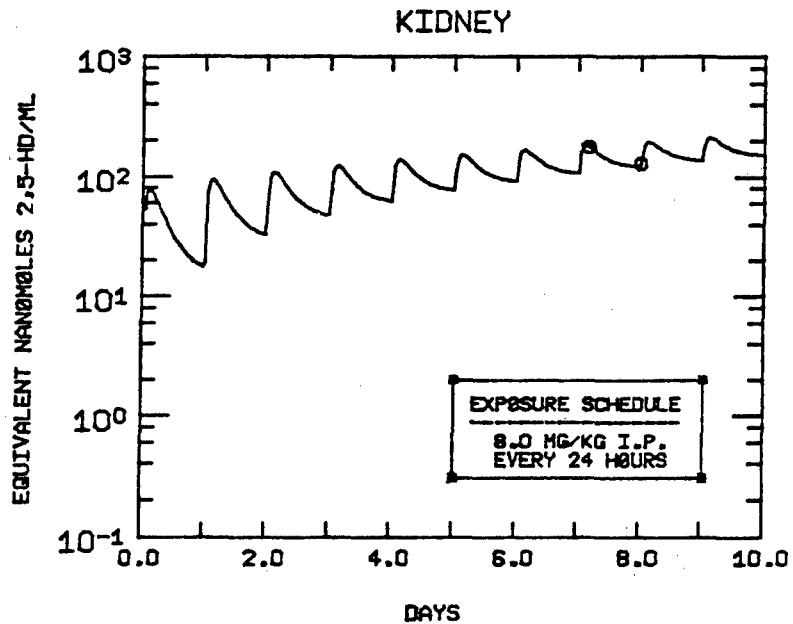


Figure 20. Model prediction of total radiolabel concentration in kidney tissue of rats following daily ip doses (8.0 mg/kg) of $[1,6-^{14}\text{C}]$ 2,5-hexanedione. Each data point represents pooled samples from two animals.

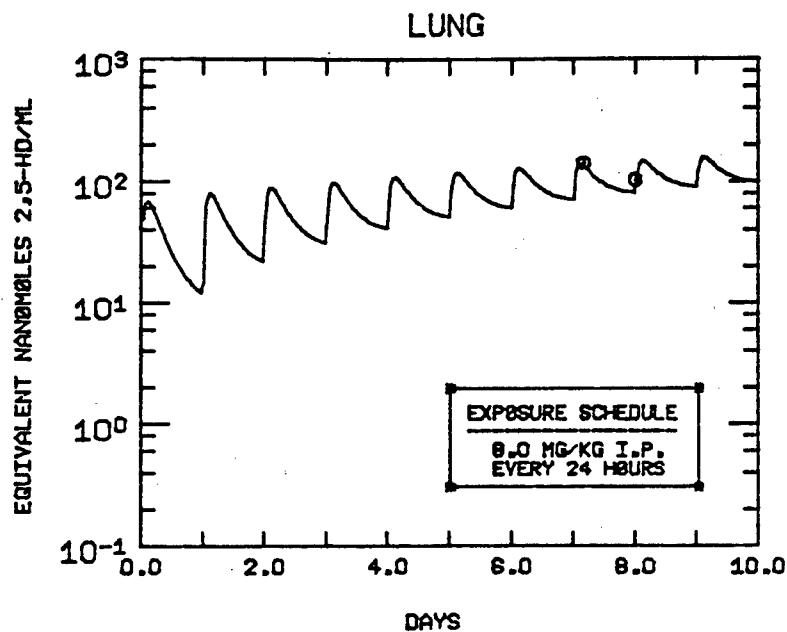


Figure 21. Model prediction of total radiolabel concentration in lung tissue of rats following daily ip doses (8.0 mg/kg) of $[1,6-^{14}\text{C}]$ 2,5-hexanedione. Each data point represents pooled samples from two animals.

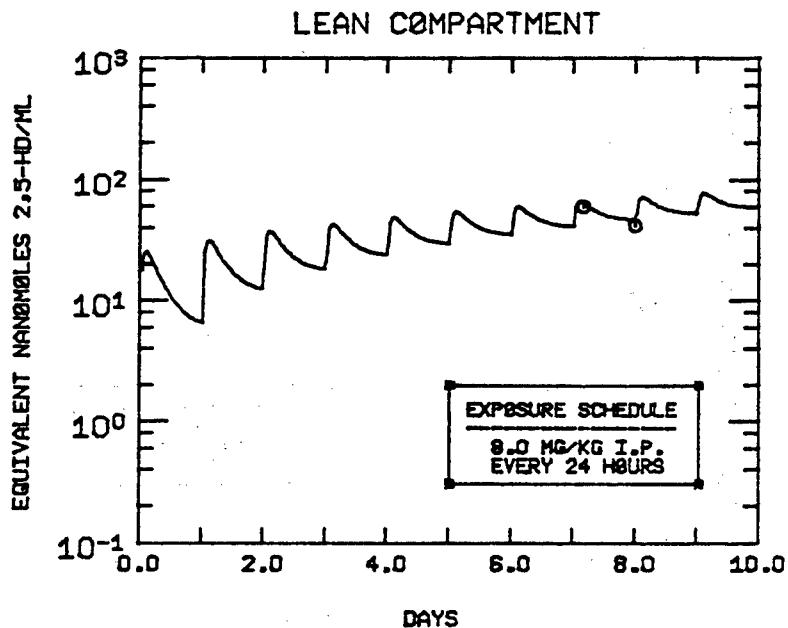


Figure 22. Model prediction of total radiolabel concentration in "lean" compartment of rats following daily ip doses (8.0 mg/kg) of $[1,6-^{14}\text{C}]$ 2,5-hexanedione. Each data point represents pooled samples from two animals.

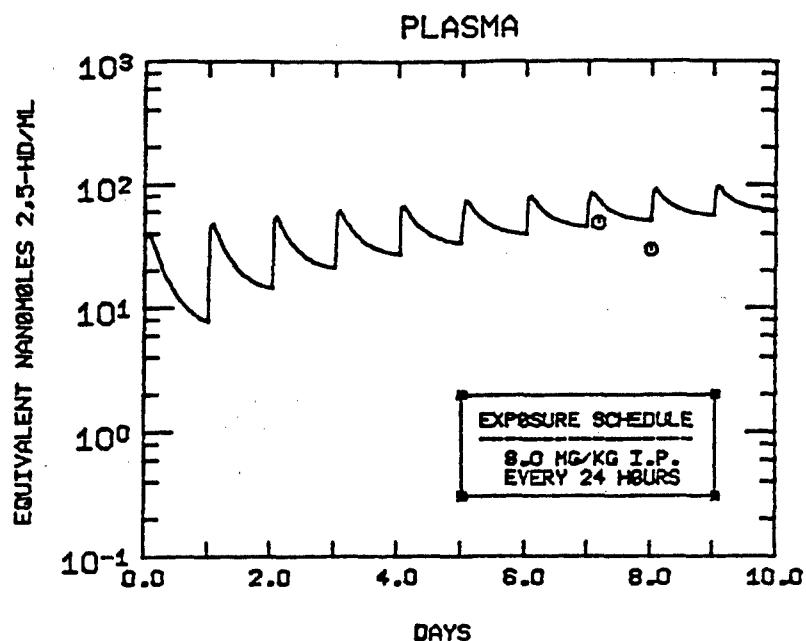


Figure 23. Model prediction of total radiolabel concentration in plasma of rats following daily ip doses (8.0 mg/kg) of $[1,6-^{14}\text{C}]$ 2,5-hexanedione. Each data point represents pooled samples from two animals.

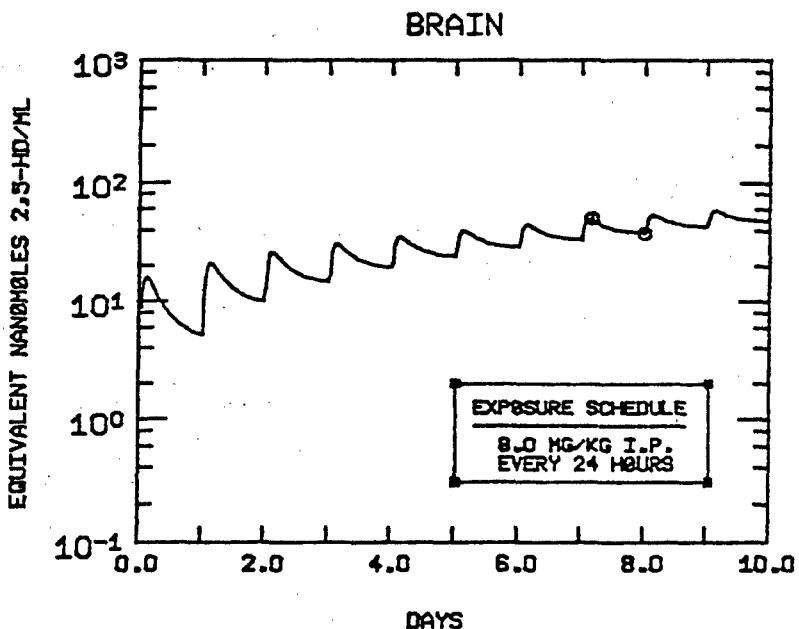


Figure 24. Model prediction of total radiolabel concentration in mixed brain of rats following daily ip doses (8.0 mg/kg) of $[1,6-^{14}\text{C}]$ 2,5-hexanedione. Each data point represents pooled samples from two animals.

For all tissues except plasma, the simulations agreed well with the data, implying that the model adequately represents the pharmacokinetic events in these tissues. The reasons for the over-prediction in the plasma compartment are not understood, but are probably related to the kinetics of ^{14}C -uptake and release by circulating plasma constituents. However, the model does at least provide a reasonable order-of-magnitude estimate of plasma concentration in the chronic dosing regimen.

CONCLUSIONS

A flow-limited physiologically-based pharmacokinetic model has been constructed to simulate ^{14}C -2,5-HD distribution and elimination as well as accounting for specific biomolecular events involving the radiolabel in individual tissue compartments. A lumping analysis served to reduce the catabolic history of ^{14}C -2,5-HD thereby making the modeling scheme mathematically tractable. Based on the agreement between simulations and experimental data for single and chronic dose exposures, this model for 2,5-HD disposition appears to present an appropriately simplified description of kinetic events involved with ^{14}C -2,5-HD catabolism and the subsequent role of released ^{14}C in intermediary metabolism.

APPENDIX

NOMENCLATURE

Symbols

A	2,5-hexanedione, i.e. parent species
B	acid soluble metabolites of 2,5-HD
C	concentration; acid insoluble (incorporated) metabolites of 2,5-HD
C_0	initial concentration
$g(t)$	dose input-function
H	hematocrit fraction in whole blood
ip	intraperitoneal
iv	intravenous
K	saturation constant
K'	equilibrium constant for $\text{CO}_2(\text{l})$ -carbonic acid system
K_a	equilibrium constant for carbonic acid-bicarbonate ion system
k_{cl}	kidney clearance

k_D	first-order rate constant for the disappearance of incorporated radiolabel from biomolecules
k_{MET}	first-order metabolic clearance rate constant for 2,5-HD
mCi	millicuries
PA	peritoneal permeability-area product
Q	plasma flow rate
R	tissue/plasma distribution ratio
T	tissue/plasma turnover ratio
t	time
V	volume
[]	concentration; radiolabel position

Subscripts

BR	brain compartment
BW	body water
EC	extra-cellular
g	gas-phase
i	tissue, non-specified
IC	intra-cellular
K	kidney compartment
L	liver compartment
l	liquid-phase
LG	lung compartment
LN	lean compartment
P	plasma compartment
PC	peritoneal cavity
SC	spinal cord compartment
SN	sciatic nerve compartment
T	tissue; total species

Superscripts

A	parent species, i.e., 2,5-HD
B	acid soluble metabolites of 2,5-HD
C	acid-insoluble metabolites of 2,5-HD
*	location of radiolabel

Compartment Mass Balances

Following are the compartmental mass balance equations that comprise the physiologically-based model. For clarity, all symbols are defined in the nomenclature section; however, it will be helpful to know that the superscripts A, B, and C refer to parent compound, soluble metabolites, and incorporated metabolites, respectively (figure 4).

Before describing the mass balance equations, it is important to note that the levels of radioactivity associated with plasma and red blood cells were similar at longer times, but that the plasma contained a significantly larger amount of radiolabel at shorter times following a single bolus dose. A mass balance on the plasma compartment was adequate in defining the distribution of parent ^{14}C -2,5-HD since this species entered the hematocrit fraction very slowly, if at all. However, a proper mass balance of the metabolite species B in tissues required using whole blood so that the ^{14}C contained in the red blood cells was accounted for. To accomplish this, the plasma flow rate to tissues was divided by (1-hematocrit fraction) in order to convert these quantities to a whole blood basis for the distribution of B. The balances for A did not require such a correction.

Plasma:

$$V_P \frac{dC_P^A}{dt} = Q_P \left(\frac{C_{LG}^A}{R_{LG}^A} - C_P^A \right) \quad (7)$$

$$V_P \frac{dC_P^B}{dt} = Q_P \left(\frac{C_{LG}^B}{R_{LG}^B} - C_P^B \right) - k_P V_P C_P^B \quad (8)$$

$$\frac{dC_P^C}{dt} = k_P (C_P^B - C_P^C) \quad (9)$$

Liver:

$$V_L \frac{dC_L^A}{dt} = Q_L (C_P^A - \frac{C_L^A}{R_L^A}) - k_M C_L^A + PA \cdot C_{PC}^A \quad (10)$$

$$V_L \frac{dC_L^B}{dt} = \frac{Q_L}{1-H} (C_P^B - \frac{C_L^B}{R_L^B}) + k_M C_L^A - k_P T_L V_L C_L^B \quad (11)$$

$$\frac{dC_L^B}{dt} = k_P T_L C_L^B \quad (12)$$

Kidney:

$$V_K \frac{dC_K^A}{dt} = Q_K (C_P^A - \frac{C_K^A}{R_K^A}) - k_{cl}^A C_P^A \quad (13)$$

$$V_K \frac{dC_K^B}{dt} = \frac{Q_K}{1-H} (C_P^B - \frac{C_K^B}{R_K^B}) - k_{cl}^B C_P^B - k_P T_K V_K C_K^B \quad (14)$$

$$\frac{dC_K^C}{dt} = k_P T_K C_K^B \quad (15)$$

Lung:

$$V_{LG} \frac{dC_{LG}^A}{dt} = Q_L \frac{C_L^A}{R_L^A} + Q_K \frac{C_K^A}{R_K^A} + Q_{BR} \frac{C_{BR}^A}{R_{BR}^A} + Q_{LN} \frac{C_{LN}^A}{R_{LN}^A} - Q_{LG} \frac{C_{LG}^A}{R_{LG}^A}$$

$$V_{LG} \frac{dC_{LG}^B}{dt} = \frac{Q_L}{1-H} \frac{C_L^B}{R_L^B} + \frac{Q_K}{1-H} \frac{C_K^B}{R_K^B} + \frac{Q_{BK}}{1-H} \frac{C_{BR}^B}{R_{BR}^B} + \frac{Q_{LN}}{1-H} \frac{C_{LN}^B}{R_{LN}^B} \quad (16)$$

$$- \frac{Q_{LG}}{1-H} \frac{C_{LG}^B}{R_{LG}^B} - k_P T_{LG} V_{LG} C_{LG}^B \quad (17)$$

$$\frac{dC_{LG}^C}{dt} = k_P T_{LG} C_{LG}^B \quad (18)$$

Brain:

$$V_{BR} \frac{dC_{BR}^A}{dt} = Q_{BR} \left(C_P^A - \frac{C_{BR}^A}{R_{BR}^A} \right) \quad (19)$$

$$V_{BR} \frac{dC_{BR}^B}{dt} = \frac{Q_{BR}}{1-H} \left(C_P^B - \frac{C_{BR}^B}{R_{BR}^B} \right) - k_P T_{BR} V_{BR} C_{BR}^B \quad (20)$$

$$\frac{dC_{BR}^C}{dt} = k_P T_{BR} C_{BR}^B \quad (21)$$

Sciatic Nerve:

$$\frac{dC_{SN}^A}{dt} = \left(\frac{Q}{V} \right)_{SN} \cdot \left(C_P^A - \frac{C_{SN}^A}{R_{SN}^A} \right) \quad (22)$$

$$\frac{dC_{SN}^B}{dt} = \frac{\left(\frac{Q}{V} \right)_{SN}}{1-H} \cdot \left(C_P^B - \frac{C_{SN}^B}{R_{SN}^B} \right) - k_P T_{SN} C_{SN}^B \quad (23)$$

$$\frac{dC_{SN}^C}{dt} = k_P T_{SN} C_{SN}^B \quad (24)$$

Spinal Cord:

$$\frac{dC_{SC}^A}{dt} = \left(\frac{Q}{V} \right)_{SC} \cdot \left(C_P^A - \frac{C_{SC}^A}{R_{SC}^A} \right) \quad (25)$$

$$\frac{dC_{SC}^B}{dt} = \frac{\left(\frac{Q}{V}\right)_{SC}}{1-H} \cdot \left(C_P^B - \frac{C_{SC}^B}{R_{SC}^B} \right) - k_P T_{SC} C_{SC}^B \quad (26)$$

$$\frac{dC_{SC}^C}{dt} = k_P T_{SC} C_{SC}^B \quad (27)$$

$$T_{SC}' = T_{SC} \cdot \left(\frac{1}{1 + \frac{C_{SC}^B}{K_{SC}}} \right) \quad (28)$$

Lean Compartment:

$$V_{LN} \frac{dC_{LN}^A}{dt} = Q_{LN} \left(C_P^A - \frac{C_{LN}^A}{R_{LN}^A} \right) \quad (29)$$

$$V_{LN} \frac{dC_{LN}^B}{dt} = \frac{Q_{LN}}{1-H} \left(C_P^B - \frac{C_{LN}^B}{R_{LN}^B} \right) - k_P T_{LN} V_{LN} C_{LN}^B \quad (30)$$

$$\frac{dC_{LN}^C}{dt} = k_P T_{LN} C_{LN}^B \quad (31)$$

Peritoneal Cavity:

$$V_{PC} \frac{dC_{PC}^A}{dt} = D \cdot g(t) - PA \cdot C_{PC}^A \quad (32)$$

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PHYSIOLOGICAL MODELING OF ENTERIC TRANSPORT

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INTRODUCTION

Uncharged, lipophilic substances are absorbed from the gastrointestinal lumen primarily by passive transport across the cell membranes of the mucosal epithelium. This mode of transport is driven by a higher thermodynamic activity in the lumen than in the blood perfusing the GI tissue. If the activity is higher in the blood than in the lumen contents, the substance will be excreted across the gut wall. Just as the capacity of the gut wall to absorb, i.e. permeability and absorptive area, varies with position along the GI tract, so should the capacity to excrete. The concentration of substances in the gut lumen is expected to vary with location because of absorption or excretion, net fluid gain or loss from the contents, and absorption of nutrients. Thus, both the driving force for exchange across the gut wall and the transport characteristics of the wall itself vary from point to point. In addition, the rate of transit of luminal contents down the GI tract varies with location. If a mathematical model of gut absorption and secretion is to contribute to the understanding of the underlying phenomena, it should reflect this inherent spatial variability.

We discuss here the development of such a model for lipophilic agents based on a physiological pharmacokinetic approach to investigating enteric transport. The model has been applied to the study of chlordcone, a chlorinated pesticide known under the trade name Kepone (Bungay et al., 1981). We summarize some of the findings from this analysis to illustrate the utility of the model.

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The principles employed were similar to those used in an earlier model for gut transport of a water-soluble drug methotrexate (Bischoff et al., 1971).

Of the components of enteric transport, GI excretion is perhaps the least studied. It is considered to play a minor rôle in the elimination of most toxic compounds in comparison with biliary and urinary excretion (Klaassen, 1975). The volumetric production rate of feces is small. Consequently, the fecal elimination rate of a substance will be low unless its concentration in the feces is high relative to blood. A lipophilic agent would generally not be concentrated in the feces if it were resistant to metabolism and conjugation. The situation with respect to excretion is exacerbated by the typically low free plasma levels because of sequestration in the body fat and/or binding to plasma constituents.

Although the elimination rate may be low, the capacity for gut excretion could be high. Potentially, the capacity could be exploited to augment excretion rates. Nondigestible material capable of binding the agent could permit high fecal concentrations relative to blood. In addition to promoting excretion across the gut wall, the adsorbent could interrupt reabsorption if the substance is excreted in the bile. The prospects for adsorbent therapy provide an additional stimulus for experimental investigation of enteric transport and hence for development of pharmacokinetic models to aid in the planning and interpretation of these studies.

MODELING THE GASTROINTESTINAL TRANSIT OF NONABSORBABLE SUBSTANCES

In physiological pharmacokinetic modeling we seek a description of the key physiological phenomena governing the disposition of substances in which as many of the parameters as possible have physical meaning and are susceptible to measurement independent of the model. At the core of our enteric model is the description of how the substance is carried along the GI tract by bulk movement of the luminal contents. This portion of our model is an extension of a concept that has been present in the pharmacokinetic literature for a long time, namely, that stomach emptying can be treated as a first order process. That is, the volumetric rate at which fluid leaves the stomach through the pylorus is proportional to the volume of the stomach contents. Alternatively, if the gastric volume is assumed constant on the average for an animal, such as the rat, which is allowed food and drink ad libitum, the volumetric emptying rate is also constant. For uniformly mixed contents, the rate at which a dose of a nonabsorbable substance leaves the stomach to enter the duodenum is also first order. That is, if the substance is administered all at once, the amount of the substance remaining in the stomach, and its concentration there, will fall exponentially in time. The instantaneous mass balance for the substance in this well-mixed stomach lumen compartment is,

$$d(V_{ST} C_{ST})/dt = -Q_{ST} C_{ST} \quad (1)$$

in which V_{ST} is the stomach volume, Q_{ST} is the volumetric rate passing through the pylorus, C_{ST} is gastric concentration, and t is time. The left-hand-side is the time derivative of the amount of the substance in the stomach while the right-hand-side expresses the rate at which the stomach is losing it. The parameters V_{ST} and Q_{ST} are, in principle, measurable for a given animal, as is the variable C_{ST} . Although the equation does not require it, for simplicity the parameters will be considered constants equal to the time-averaged values of these quantities. Equation (1) can then be rewritten as,

$$dC_{ST}/dt = -C_{ST}/T_{ST} \quad (2)$$

with $T_{ST} = V_{ST}/Q_{ST}$ being the time constant for stomach emptying.

One would similarly write a single compartmental mass balance for the substance in each of the other luminal segments of the GI tract: small intestine, cecum, and colon. However, the assumption of a uniform, instantaneous concentration throughout the small intestine lumen would be a crude approximation considering the variation in concentration along this organ that has been observed experimentally (e.g. Sikov et al., 1969). Longitudinal mixing or dispersion does occur, but the transit of nonabsorbable tracer substances exhibits a significant degree of pulse or plug-like character. To simulate this we have subdivided the small intestine lumen longitudinally into a series of well-stirred compartments. The extent of longitudinal mixing decreases with the number of compartments in series.

For the j^{th} subcompartment the mass balance becomes

$$V_{L_j} \frac{dC_j}{dt} = \frac{V_{L_{j-1}} C_{j-1}}{T_{j-1}} - \frac{V_{L_j} C_j}{T_j} \quad (3)$$

where we have already substituted $Q_j = V_{L_j}/T_j$ for the flow rate of the contents leaving the j^{th} subcompartment. Here T_j is average residence or transit time for the contents within this subcompartment. The difference between Q_j and the rate at which the contents

are entering the j^{th} subcompartment from the one just preceding it, $Q_{j-1} = V_{L_{j-1}} / T_{j-1}$, is the net rate of fluid secretion or absorption for the j^{th} subsegment. Again, we are taking the volumes, flow rates, and time constants to be the time-averaged values of these quantities. Equation (3) can be used similarly to represent portions of the remainder of the GI tract. We have used one such equation each to describe the cecum and large intestine.

The sum of all the volumes for the subcompartments is equal to the small intestine lumen volume. The modeler has at his discretion the number of subcompartments, the distribution of the total lumen volume among the subcompartments, and the respective transit times. One chooses values for these parameters such that the simultaneous solution of the set of equations (2) and (3) simulates the spatial variation in processes such as longitudinal transit and mixing and net fluid shifts across the gut wall. Depending upon how much experimentally-derived information is available, one can determine the parameter values in various ways. For our rat model we adjusted our parameter values to obtain simulations corresponding to literature data from the measurement of intact rats of the transit of orally administered, nonabsorbable, radioactive tracers obtained by Sikov et al. (1969). The points in Figure 1 are derived from the data. The curves in this figure have been generated from the model equations showing the variation in tracer content over time in the stomach, three sequential segments of the small intestine, cecum, large intestine, and cumulative feces.

MODELING GASTROINTESTINAL ABSORPTION, METABOLISM, AND EXCRETION

In formulating the model equations in the previous section the gut wall was considered impermeable. The equations can be modified to permit the substance to enter and leave the lumen subcompartments by transport across the mucosal epithelium. Various concepts have been employed for modeling absorption from the gut lumen as discussed by Winne (1978). The utility of the model depends upon the kinds of experimental measurements to be employed. Since we measured lumen contents, gut tissue, and blood levels as a function of time, we chose what we considered to be the simplest model which incorporates these quantities as the variables.

We assume that the resistance to exchange between the lumen contents and blood can be localized in two interfacial barriers: a mucosal barrier between the lumen and the tissue, and a capillary barrier between the tissue and the blood. The gut tissue and the blood within the gut wall are treated as separate compartments

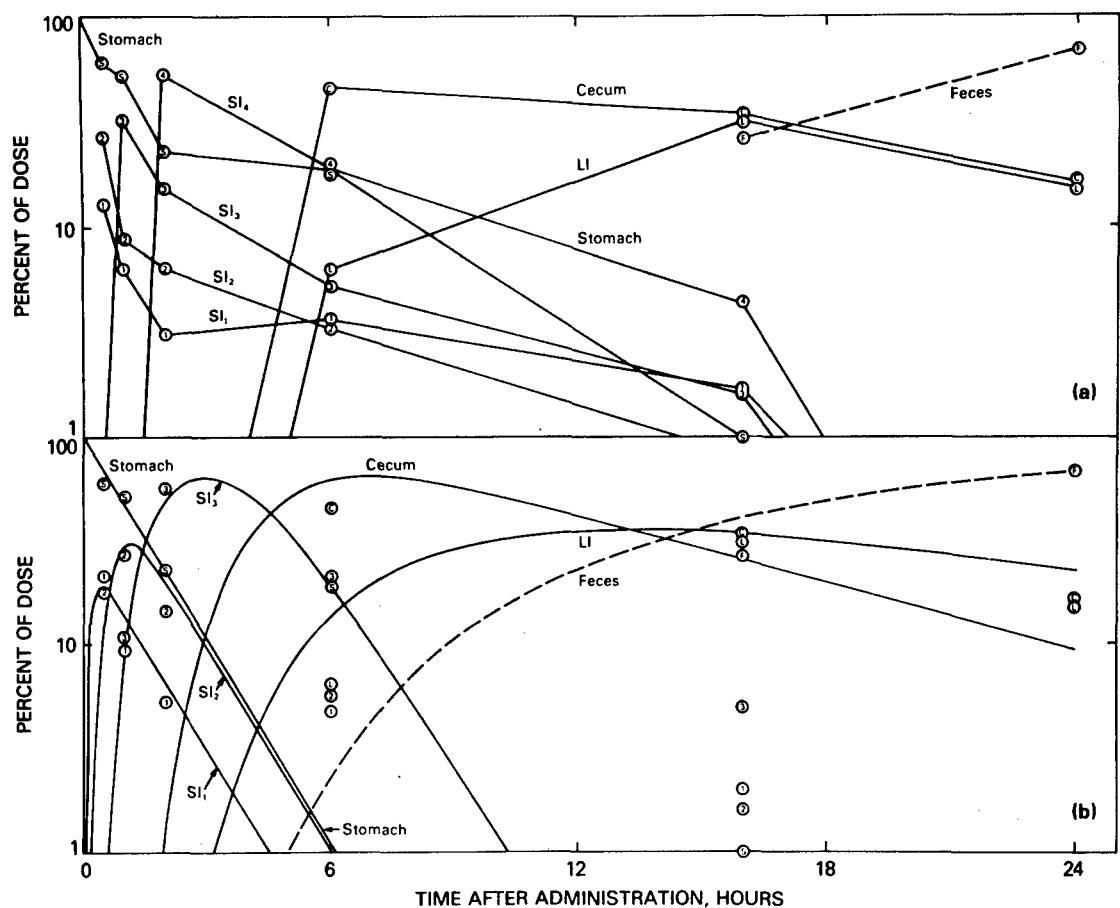


Figure 1. Transit along gastrointestinal tract of trace amounts of nonabsorbable marker in the non-fasted adult rat. Marker administered by gavage at time zero. Data points: S, stomach; 1, 2, 3, 4, series segments of small intestine (SI); C, cecum; L, large intestine (LI); F, unreported radioactivity assumed to be excreted in feces. **a.** Data of Sikov, Thomas, and Mahlum (1969) plotted as percent of radioactivity from an equilibrium solution of ^{106}Ru - ^{106}Rh chloride retained in each lumen compartment against elapsed time. Small intestine was segmented in quartiles of equal length. Lines connect sequential data points from a specific gut segment. **b.** Replot of data from (a) except radioactivity in small intestine reapportioned for three segments of equal length. Solid curves were generated by numerical solution of the gut transport series compartmental model for an impermeable gut lumen wall with stomach emptying time, $T_S = 80$ min., and lumen transit times: $T_1 = 20$ min., $T_2 = 40$ min., $T_3 = 150$ min., $T_C = 480$ min., $T_{LI} = 480$ min.

with all of the tissue being interposed between the lumen and the blood. As with the lumen, the gut tissue and gut blood compartments are subdivided longitudinally along the GI tract. The j^{th} lumen, tissue, and blood subcompartments are shown schematically in Figure 2 with the respective barrier for exchange between them shown as dotted lines.

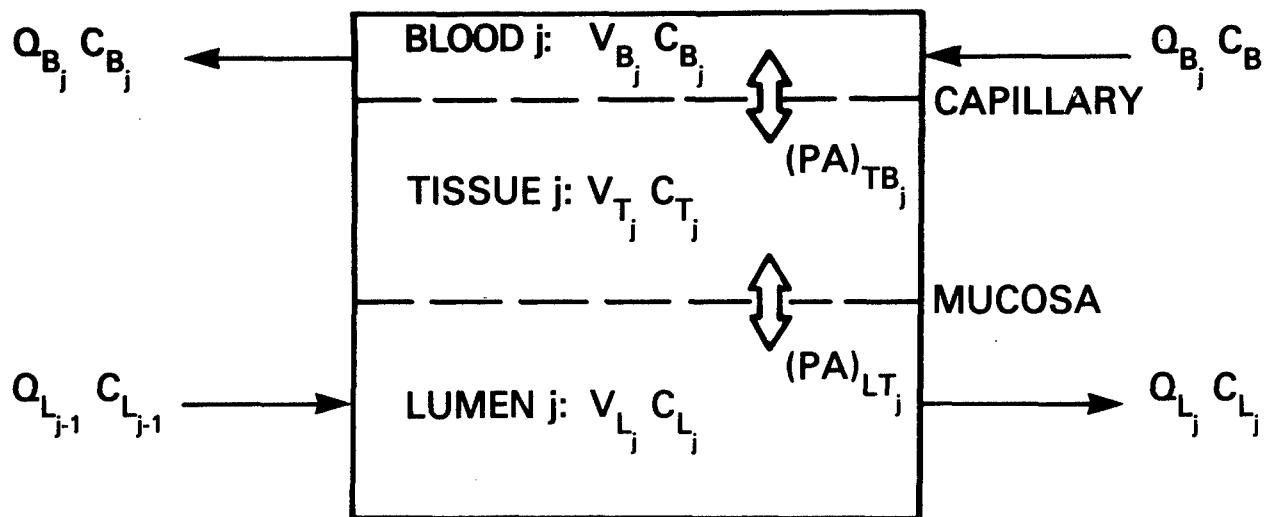


Figure 2. Schematic design of the j^{th} segment of the gut showing blood, tissue and lumen compartments separated by transport barriers. Symbols: PA = permeability-area products characterizing barriers; C = compartment concentration; Q = volumetric flow rate; V = compartment volume. Subscripts: B = arterial blood; B_j = venous blood; L_j = lumen; T_j = tissue; LT_j = lumen-to-tissue; TB_j = tissue-to-blood; j = stomach, segments of small intestine, cecum, and large intestine. Not shown are the equilibrium distribution coefficients, $R_{LT,j}$ and $R_{TB,j}$.

The blood subcompartments are perfused in parallel, that is, arterial blood enters each subcompartment and the effluent streams merge to form the portal vein flow. Each blood subcompartment is considered well mixed with a concentration equal to its venous level, C_{Bj} .

Each tissue subcompartment is likewise taken to be well mixed with a uniform concentration, C_T . The resistance to transport into or out of the tissue subcompartment is embodied in the products of permeability, P , multiplied by exchange area, A . The transport is assumed to be symmetric, that is, the PA product is

the same for absorption or excretion. A PA product can be considered a single parameter with a different value, $(PA)_{TB_j}$, for each capillary and $(PA)_{LT_j}$, for each mucosal interface in each subsegment of the GI tract.

The appropriate mass balances for the blood and tissue subcompartments, together with the modified lumen balance, are:

$$\text{Blood: } V_{B_j} (dC_{B_j} / dt) = Q_{B_j} (C_B - C_{B_j}) + (PA)_{TB_j} (C_{T_j} / R_{TB_j} - C_{B_j}) \quad (4)$$

$$\text{Gut tissue: } V_{T_j} (dC_{T_j} / dt) = (PA)_{TB_j} (C_{B_j} - C_{T_j} / R_{TB_j}) \\ \quad + (PA)_{LT_j} (C_{L_j} / R_{LT_j} - C_{T_j}) \quad (5)$$

$$\text{Gut lumen: } V_{L_j} (dC_{L_j} / dt) = V_{L_{j-1}} C_{L_{j-1}} / T_{L_{j-1}} - V_{L_j} C_{L_j} / T_{L_j} \\ \quad + (PA)_{LT_j} (C_{T_j} - C_{L_j} / R_{LT_j}) - V_{L_j} K_j C_j \quad (6)$$

The last term in equation (6) indicates a way to account for metabolism in the gut lumen, as for example by intestinal bacteria. This expression assumes that metabolism can be represented as a disappearance rate which is proportional to the concentration of the substance; however, more complex kinetic expressions can be used if required. The parameter K_j is then a first order reaction rate constant for a unit mass of contents. In order to simplify the present treatment this metabolism term will be neglected. It should be pointed out, however, that in this physiologic pharmacokinetic approach, metabolite transport can likewise be described by writing balance equations for the metabolite, analogous to equations (4) through (6).

The tissue and lumen volumes, V_{T_j} and V_{L_j} , were obtained from measurements on excised material. Blood volumes, V_{B_j} , and flow rates, Q_{B_j} , were calculated from the tissue volumes based on proportionality factors from the literature. The lumen transit times

were evaluated from gut transit experiments as described in the previous section. These parameters are particular to the animal and independent of the substance administered. The determination of values for parameters $(PA)_{LT_j}$, $(PA)_{TB_j}$, R_{LT_j} , and R_{TB_j} requires additional information derived from experiments with the substance of interest. The R parameters are the lumen-to-tissue or tissue-to-blood concentration ratios at equilibrium.

In principle, the PA and R parameters can be evaluated from various experiments. In applying the model to chlordcone transport we used data from the early transient distribution phase of experiments in which the chlordcone was administered to rats. In one batch of animals the pesticide was administered by gavage in order to emphasize absorptive processes. In another batch, the chlordcone entered the GI lumen by excretion following intravenous administration. The numerical solutions to the model equations were compared to plots of the measurements of blood, tissue and lumen concentrations. The PA and R values were adjusted until reasonable agreement was obtained between the experimental data and the computer-generated concentrated versus time curves.

Along with the above mass balance equations for the gut, we incorporated analogous balances for the other organs which play a significant role in the distribution of chlordcone: fat, liver, muscle, and skin. Thus the detailed enteric transport model was embedded in a larger physiological pharmacokinetic model. Whole-body distribution studies were also performed for chlordcone. The particulars can be found in Bungay et al. (1981). Figure 3 shows the schematic flow diagram for the whole-body model. The model introduces several additional aspects of the enteric transport system: biliary secretion and the possibility of incorporating metabolic conversion in the liver or other organs. For illustration, Figure 4 shows the time course of chlordcone levels in the lumen compartments during the four hours following oral administration. As before the curves were generated from the model while the points represent experimental data.

DISCUSSION

From measurement during the distribution transients in the rat following oral and intravenous administration, combined with the mathematical model outlined in the two previous sections, we have obtained estimates of chlordcone permeability-area products for

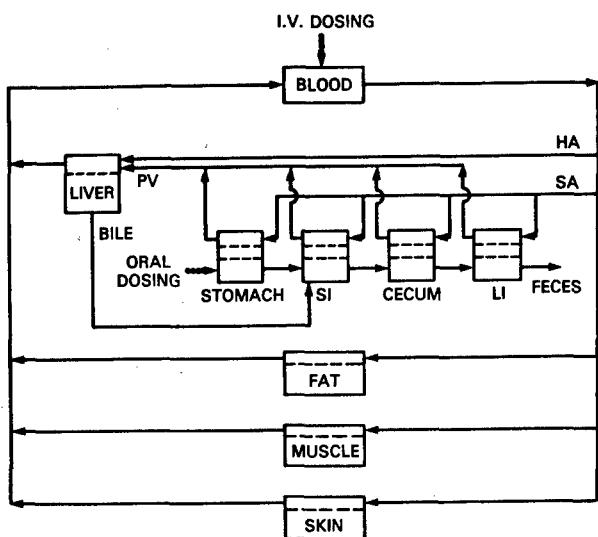


Figure 3. Schematic diagram for whole-body physiological pharmacokinetic model for chlordecone. Solid lines with arrows denote flows of body fluids: blood, bile, gut contents, and feces. Dotted lines with arrows point to compartments to which chlordecone is administered. Dashed lines between compartments indicate transport barriers characterized by permeability-area products. Small intestine is further divided into three segments (not shown). Symbols: HA = hepatic artery; PV = portal vein; SA = splanchnic artery; LI = large intestine; SI = small intestine.

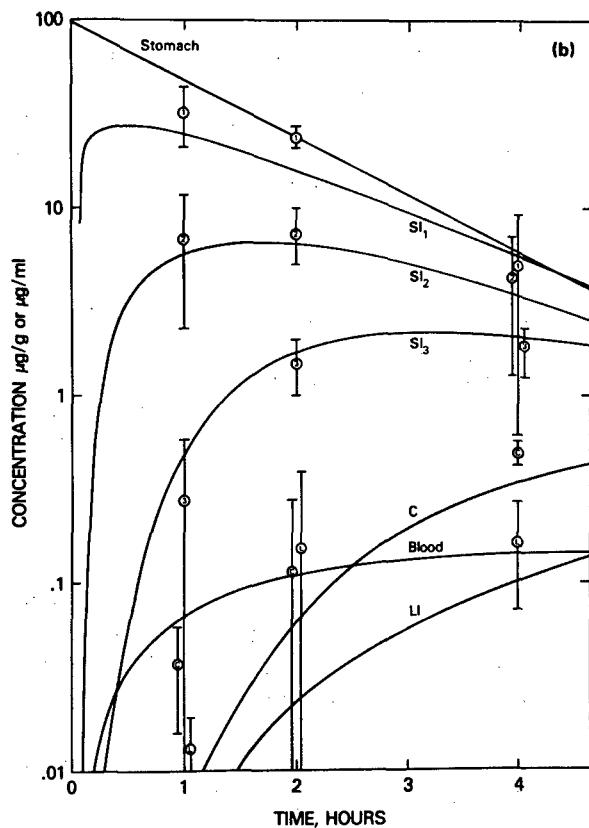


Figure 4. Time course of ^{14}C -chlordecone in the gastrointestinal lumina of rats following a single oral administration of 1 mg/kg. Data points: S, stomach; 1, 2, 3, series of segments of small intestine (SI); C, cecum; L, large intestine (LI); B, blood. Lines were generated by numerical solution of the pharmacokinetic model.

the mucosal and capillary interfaces. It has not been substantiated whether chlordenecone is metabolized in the rat. Chromatographic analysis of bile and feces samples suggested that these experiments were of sufficiently short duration (4 hours or less) that the proportion of parent compound converted to metabolites was small. The PA values obtained thus apply to the parent compound. It can be expected that the permeability of the gut wall to metabolic products, if they exist, would be different. Boylan et al. (1979) isolated chlordenecone alcohol, in addition to the parent compound, from bile and stool samples of a victim of chlordenecone poisoning. Fariss et al. (1980) determined that conjugates of chlordenecone and chlordenecone alcohol were present in the human bile. However, no metabolic products of chlordenecone have been isolated from blood samples. In principle, if concentrations of metabolites are measured, their behavior can be included in the model by the addition of appropriate mass balances.

With the aid of the mathematical model and the PA values for the parent compound we are able to speculate as to the potential of adsorbent therapy. As indicated in the introduction, nondigestible adsorbents can be administered orally to increase the rate of fecal elimination of toxic substances. For example, Cohn et al. (1978) reported on a clinical trial involving 22 patients suffering from chlordenecone poisoning. The average half-time of chlordenecone blood levels in these patients was reduced approximately 50% from 165 days to 80 days by administration of cholestyramine. Similar results were obtained by these investigators in studies with rats (Boylan et al., 1978). However, cholestyramine does not selectively bind chlordenecone. One would like to ascertain whether the elimination rate could be further increased by employing a selective adsorbent. Toward this end we have used the enteric model to estimate a measure of the maximum rate of excretion achievable with an adsorbent.

The maximum rate of excretion across the gut wall would occur if the adsorbent maintained the thermodynamic activity of the toxic agent effectively zero throughout the lumen of the GI tract. The excretion would then be limited either by the rate of delivery of the substance to the GI tissue by the blood, or by the resistance to transport across the gut wall, or to both if these factors are comparable.

Based on our estimates of PA and blood flow in the rat, gut excretion of chlordenecone would appear to be limited by the diffusional resistance. We calculated the maximum clearance (excretion rate divided by blood concentration) by transport across the gut wall in 270 g rats to be 23 ml/hr (Bungay et al., 1981). Measurements of biliary excretion of chlordenecone in rats yielded a clearance by this route of 5 ml/hr. Summing the gut and biliary contributions leads to a predicted maximum clearance by elimination in the stool of 28 ml/hr. Values for clearances from rats not treated

with cholestyramine varied over the range of 1 to 4 ml/hr suggesting that an optimal adsorbent might be able to increase the excretion rate sevenfold rather than the twofold increase observed with cholestyramine.

Using pharmacokinetic scaling principles, one can extrapolate these predictions to man. We assume that the permeability characteristics of the human gut for chlordcone will be similar to those of the rat. External body surface area increases approximately in proportion to the 2/3 power of body weight. Assuming this scaling pertains as well to the exchange area of the gut, we estimate a maximum gut wall clearance in humans of 940 ml/hr. Combined with a value for chlordcone biliary clearance of 50 ml/hr calculated from human bile cannulation data (Cohn et al., 1978), this estimate yields a maximum fecal clearance of about 1000 ml/hr. In comparison, the cholestyramine treatment produced an average blood half-time equivalent to a far lower clearance of 80 ml/hr. If chlordcone can be considered as a marker toxic substance representing the halogenated hydrocarbons, these results suggest the capacity of the gut for excretion could be exploited to much greater degree than it has.

The above analysis is offered as but one example of the utility of enteric transport modeling. Such calculations, however, will remain speculative until studies of the underlying processes provide independent measurement of the parameters. Physiological pharmacokinetic models, such as the one described, should aid future understanding of the role of the gut in the disposition of drugs and environmental contaminants.

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**PHYSIOLOGICAL PHARMACOKINETIC MODELS FOR THE
INHALATION OF GASES AND VAPORS***

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INTRODUCTION

The most fundamental premise of experimental toxicology is that coherent relationships exist between the intensity of a particular toxic effect and the concentration of toxic chemical at the target tissue in the organism. Frequently, and this is especially true with vapors of solvent chemicals, the parent compound is relatively innocuous and toxicity is related to formation of reactive metabolites. For these chemicals, we expect the intensity of the toxic effect to be more directly related to the amount of parent chemical metabolized. Rates of metabolism of inhaled xenobiotics in target tissues can be related to circulating concentrations of the inhaled chemical by understanding the physiological and biochemical factors that control biotransformation rates *in vivo*.

In most inhalation toxicity experiments we do not have direct data regarding inhalant concentrations at internal target tissues. Instead, we know simply inhaled concentration and duration of exposure. A major contribution of pharmacokinetic analysis is to predict the relationship between inhaled concentration and concentration at target tissues. In addition, these analyses can tell us how quickly internal concentrations are achieved after beginning an exposure and how rapidly they are diminished when the exposure ends.

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As a distinct toxicology subdiscipline, inhalation pharmacokinetics is the study of the uptake, distribution, metabolism, and elimination of potentially toxic chemicals by test animals. Concentrations of the inhaled chemical are determined in blood, tissue, or excreta at various times during and after exposure. These time course curves are fitted based on some idealized, mathematical reconstruction of the physiological system. Two frequently used mathematical approaches are compartmental modeling and physiological modeling. Kinetic analyses for oral and intravenous administration of drugs have most often been done using the former procedure and various texts describing compartmental analysis are available (Gibaldi and Perier, 1975). Recently, compartmental models have been used to describe the uptake and metabolism of various gases and vapors (Andersen et al., 1980; Bolt et al., 1977). A schematic of a 3-compartment model is shown in Figure 1. These compartments have no direct anatomic analog, but the models can often be solved explicitly to give estimates of the various intercompartmental rate constants.

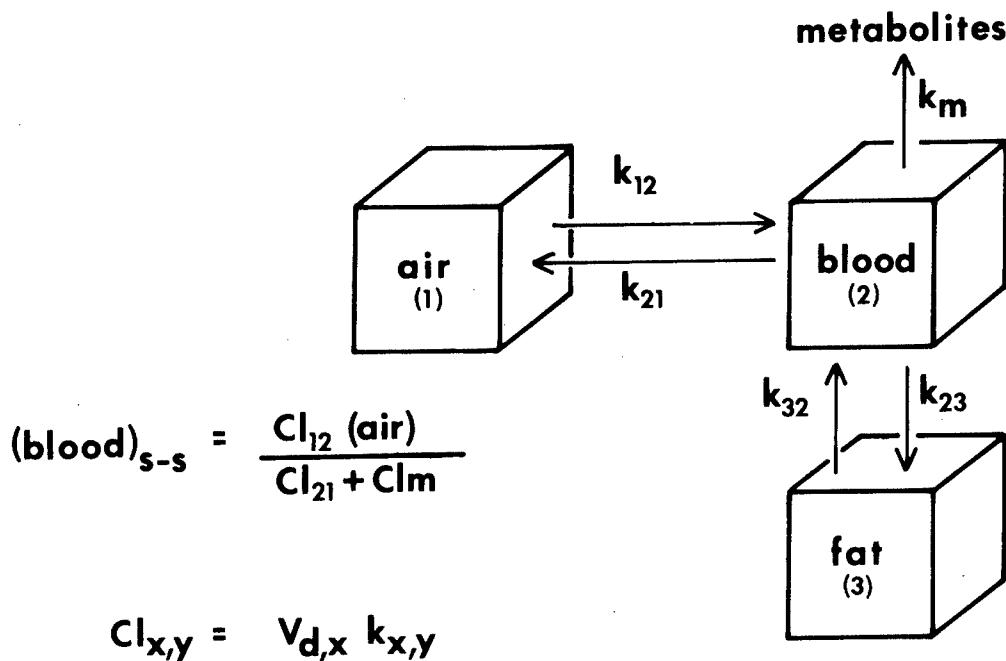


Figure 1. A three compartment pharmacokinetic model of the metabolism of inhaled gases and vapors. Metabolic clearance from the central compartment is the rate constant for metabolism, k_m , times the compartmental volume, V_2 . Other clearance pathways are exhalation ($Cl_{23} = k_{23}V_2$) and tissue loading ($Cl_{23} = k_{23}V_2$). Concentration in the central blood containing compartment will be equal to the product of inhaled concentration times the solubility coefficient only when $Cl_{21} \gg (Cl_m + Cl_{21})$.

Because the compartments are not well defined biologically, results in one species cannot be readily extrapolated to predict behavior in another, untested species. In contrast, physiological models (Figure 2) describe the animal in terms of particular organs with associated blood flows, volumes, and partition coefficients (Fiserova-Bergerova et al., 1974, 1980; Bischoff et al., 1971). Physiological models are more complex mathematically, but extrapolation to untested species is possible based on differences in physiology and biochemistry between the tested and untested species (Dedrick and Bischoff, 1980). This extrapolative strategy is termed animal scale-up.

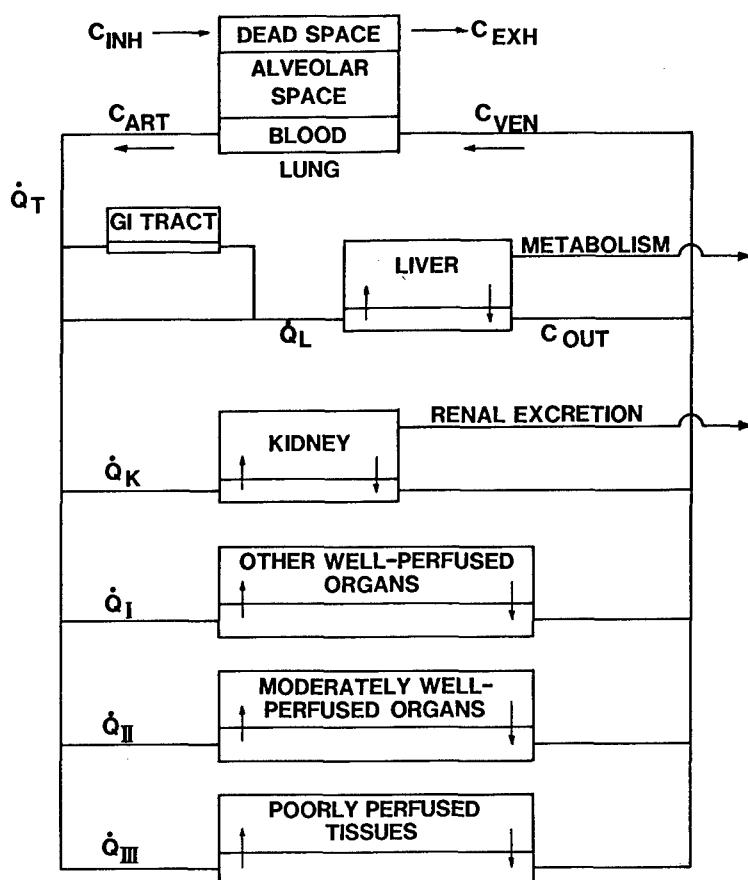


Figure 2. A physiologically based toxicokinetic model for metabolism of inhaled gases and vapors. The model is characterized by two metabolizing organs, liver and kidney, and other organs are lumped according to their perfusion/solubility characteristics.

This paper describes contributions of physiological models to understanding inhalation pharmacokinetics. The first section discusses modeling the uptake, distribution, equilibrium, and elimination of gases and vapors which are resistant to biotransformation. A second section analyzes the steady-state behavior of gases and vapors which are readily metabolized *in vivo*. The last part of the paper describes a preliminary attempt to construct a generally applicable, flow-limited physiological model of inhalation based on the scheme in Figure 2. Abbreviations and symbols used in this paper are defined in Appendix A.

METABOLICALLY INERT GASES AND VAPORS

Equilibrium Condition

For a chemical that is unaffected by the body's metabolic machinery, inhalation will eventually produce a true equilibrium condition. The concentration in blood at equilibrium will be related to the inhaled concentration times the solubility coefficient for the chemical in blood.

$$C_{\text{blood}} \equiv C_{\text{art}} \equiv C_{\text{ven}} \equiv S_b \cdot C_{\text{inh}} \equiv S_b \cdot C_{\text{alv}} \quad (1)$$

This says simply that no chemical is removed during circulation ($C_{\text{art}} \equiv C_{\text{ven}}$) and no net uptake occurs ($C_{\text{inh}} \equiv C_{\text{alv}}$). The average solubility of chemical in individual organs will be related to blood concentrations by average organ/blood solubility coefficients. For instance,

$$C_{\text{liver}} \equiv S_{\text{liver:blood}} \cdot C_{\text{blood}} \quad (2)$$

Similar equations can be written for each organ at equilibrium.

With inert gases, important issues are how quickly equilibrium is achieved and what physiological factors determine the time-course of equilibration. In a series of seminar papers, Haggard (1924a,b,c) developed and evaluated a physiological model for describing the uptake distribution and elimination of diethyl ether.

Among the equations derived was one to estimate the grams of an inert substance absorbed in the first minute of inhalation - equation (3).

$$Amt_{1 \text{ min}} = \frac{\dot{Q}_t C_{inh} S_b \dot{V}_{alv}}{\dot{Q}_t S_b + \dot{V}_{alv}} \quad (3)$$

Riggs (1970) examined the dynamics of the simple case where the body was considered a single homogeneous unit with some volume of distribution (V_d) for the inhaled substance (Figure 3). At equilibrium the total amount of chemical in the body would be:

$$Amt_{\infty} = V_d C_{blood} = V_d C_{inh} S_b \quad (4)$$

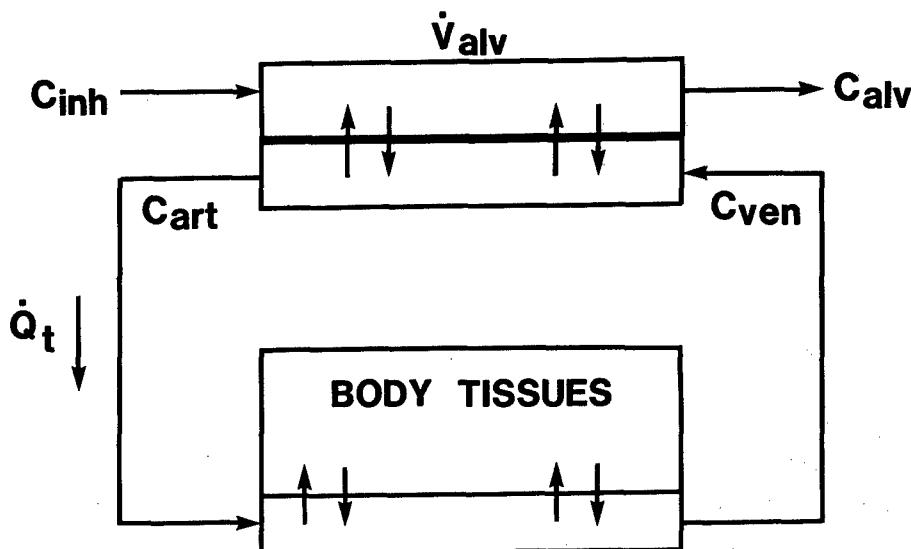


Figure 3. A simplified physiological model in which the body is assumed to be a single homogeneous unit.

The equation relating the amount in the body at any time, t , was:

$$A_{mt} = V_d S_b C_{inh} - V_d S_b C_{inh} \exp - \frac{\dot{V}_{alv} \dot{Q}_t t}{V_d (S_b \dot{Q}_t + \dot{V}_{alv})} \quad (5)$$

For small values of t , this equation is equivalent to equation (3). The rate constant for equilibration in this model is:

$$k = \frac{\dot{V}_{alv} \dot{Q}_t}{V_d (S_b \dot{Q}_t + \dot{V}_{alv})} \quad (6)$$

Consider the two extremes of a very poorly soluble vapor ($S_b \ll 1$) and a highly soluble vapor ($S_b \gg 1$). For the former case:

$$k \approx \frac{\dot{Q}_t}{V_d} \quad (7)$$

and blood flow limits uptake. For the latter case:

$$k \approx \frac{\dot{V}_{alv}}{V_d S_b} \quad (8)$$

and alveolar ventilation and solubility are expected to regulate uptake or elimination of a soluble vapor.

This is, of course, a simplified model which does not account for perfusion and solubility idiosyncrasies of individual organs. Despite its limitations, the simplified description indicates those physiological factors important for uptake (and similarly elimination) of inert gases and vapors. The reader is directed to other discussions of the dynamics of uptake that include direct consideration of individual organs or groups of organs in relation to approach to equilibrium (Fiserova-Bergerova et al., 1974, 1980; Riggs, 1970). One constant expectation in all models for uptake of inert gases and vapors is that the shape of the time-course curves will be invariant with respect to inhaled concentration (Figure 4). This is true whether we consider multiple organs individually or some homogeneous body model.

METABOLICALLY INERT GAS; $S_b=10$

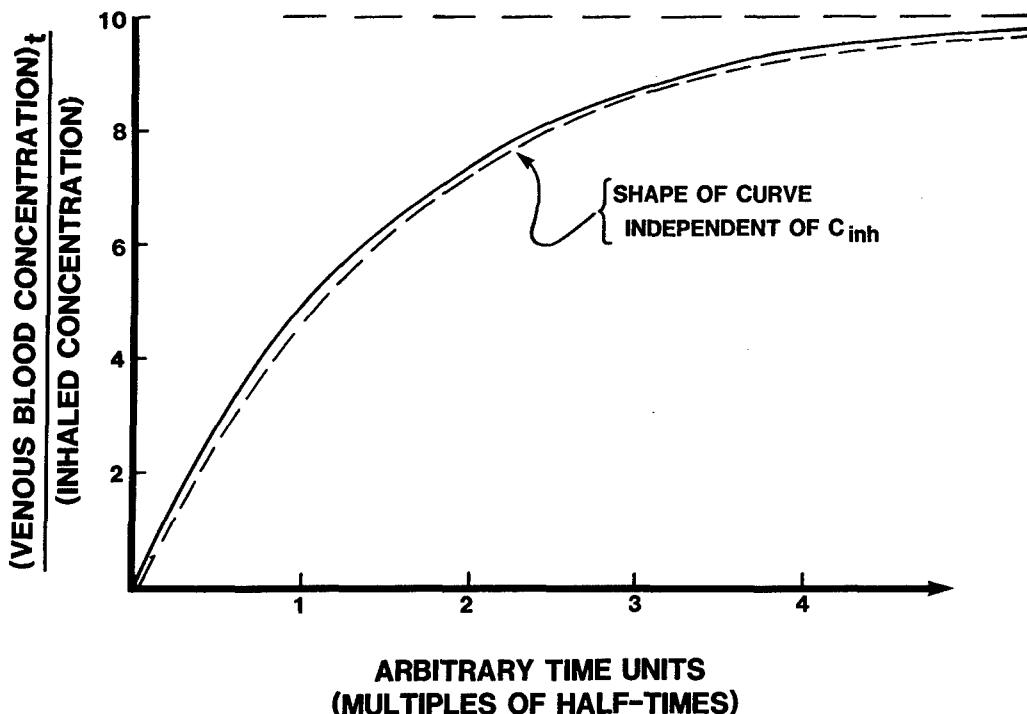


Figure 4. Schematized equilibration curves for the homogeneous body model for a metabolically inert gas or vapor. The curves are drawn for a substance with a blood:gas solubility coefficient of 10.

METABOLIZED GASES AND VAPORS

Most inhaled gases and vapors are metabolized to some extent after absorption by inhalation. Biotransformation reactions of xenobiotic metabolism are enzyme catalyzed and show capacity-limited phenomena at high concentrations. But whether we discuss low concentrations where reactions are nearly first-order, or high concentrations where reactions appear zero-order due to enzyme saturation, certain relationships will be valid. A metabolized chemical will not reach equilibrium regardless of the length of exposure. Instead, a steady-state will be reached. This occurs because C_{ven} will always be less than C_{art} , due to metabolic loss in various

tissues, and C_{alv} will be less than C_{inh} , as pulmonary uptake is driven by depletion of arterial concentrations due to metabolism. The conservation equation at steady-state is:

$$\dot{\mu}_{s-s} = \dot{V}_{alv} (C_{inh} - C_{alv}) = \dot{Q}_t (C_{art} - C_{ven}) \quad (9)$$

Andersen (1981) redefined certain of these variables and solved equation (9) for the expected blood:gas concentration ratio at steady-state ($S_{b,eff}$):

$$S_{b,eff} = \frac{C_{art}}{C_{inh}} = \frac{\dot{V}_{alv} S_b}{\dot{V}_{alv} + \dot{Q}_t S_b E_t} = \frac{S_b}{1 + \frac{\dot{Q}_t}{\dot{V}_{alv}} E_t S_b} \quad (10)$$

In this relationship, E_t is the systemic extraction ratio, $(C_{art} - C_{ven})$ divided by C_{art} . Again, similarities between this equation and equations (3) and (5) should be obvious.

The exact numerical value of E_t depends on the particular gas or vapor and its concentration in blood perfusing the metabolizing organs. Metabolism of a variety of vapors appears to be limited by organ perfusion at low concentrations (Table 1). Essentially, this means that all chemical going to the metabolizing organ, presumably the liver, is metabolized at low concentrations. Since the liver receives about 25% of cardiac output, E_t is about 0.25 when hepatic metabolism is perfusion limited. As arterial concentration increases, hepatic enzyme systems become saturated and perfusion is no longer rate limiting. E_t then should diminish with increasing concentration. Because E_t is variable, the steady-state blood:gas concentration ratio [equation (10)] will also vary with C_{inh} . Thus, the apparent rate constant for approach to equilibrium is also expected to vary with C_{inh} . (Simplistically, $S_{b,eff}$ replaces S_b in equation (5).) This behavior is schematized in Figure 5. Furthermore, the steady-state arterial blood concentration will not be a linear function of inhaled concentration as it is for metabolically inert gases and vapors. The complex dependence of C_{art} on C_{inh} observed with chemicals such as styrene (Ramsey and Young, 1978) and vinyl toluene (Karbowski et al., 1980) is just an expected consequence of perfusion-limited metabolism (Figure 6).

TABLE 1. INHALED VAPORS AND GASES FOR WHICH PERfusion OF THE METABOLIZING ORGAN APPEARS RATE-LIMITING* FOR METABOLISM IN RATS AT LOW INHALED CONCENTRATIONS

N-Hexane	Halothane
Cyclohexane†	Chlorobromomethane
Heptane†	Methylene chloride
Cycloheptane†	Vinyl bromide
Methylcyclohexane†	1,1-Dichloroethylene
2-Hexane	Vinyl chloride
Benzene	Trichloroethylene
Vinylmethylether	Vinyl fluoride
Cis-1,2-dichloroethylene	Vinylidene fluoride
Trans-1,2-dichloroethylene	Perchloroethylene

* Evidence for perfusion-limitation reviewed for most chemicals by Andersen (1981).

† Unpublished experiments (Kroon, Woods, and Andersen, 1980).

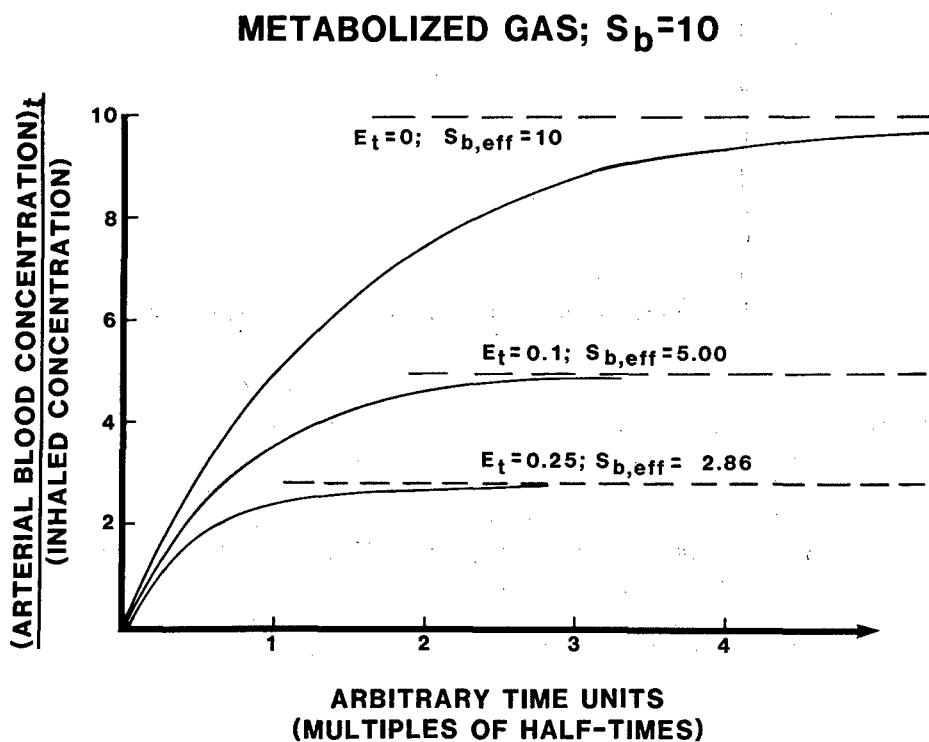


Figure 5. Schematized equilibration curves for a metabolized gas or vapor. The y-axis is a ratio of arterial to inhaled concentration. The three curves are for varying values of the systemic extraction (E_t). They indicate that both the time course of equilibration and the final steady-state blood:gas concentration ratio are affected by varying E_t with a soluble gas ($S_b = 10$).

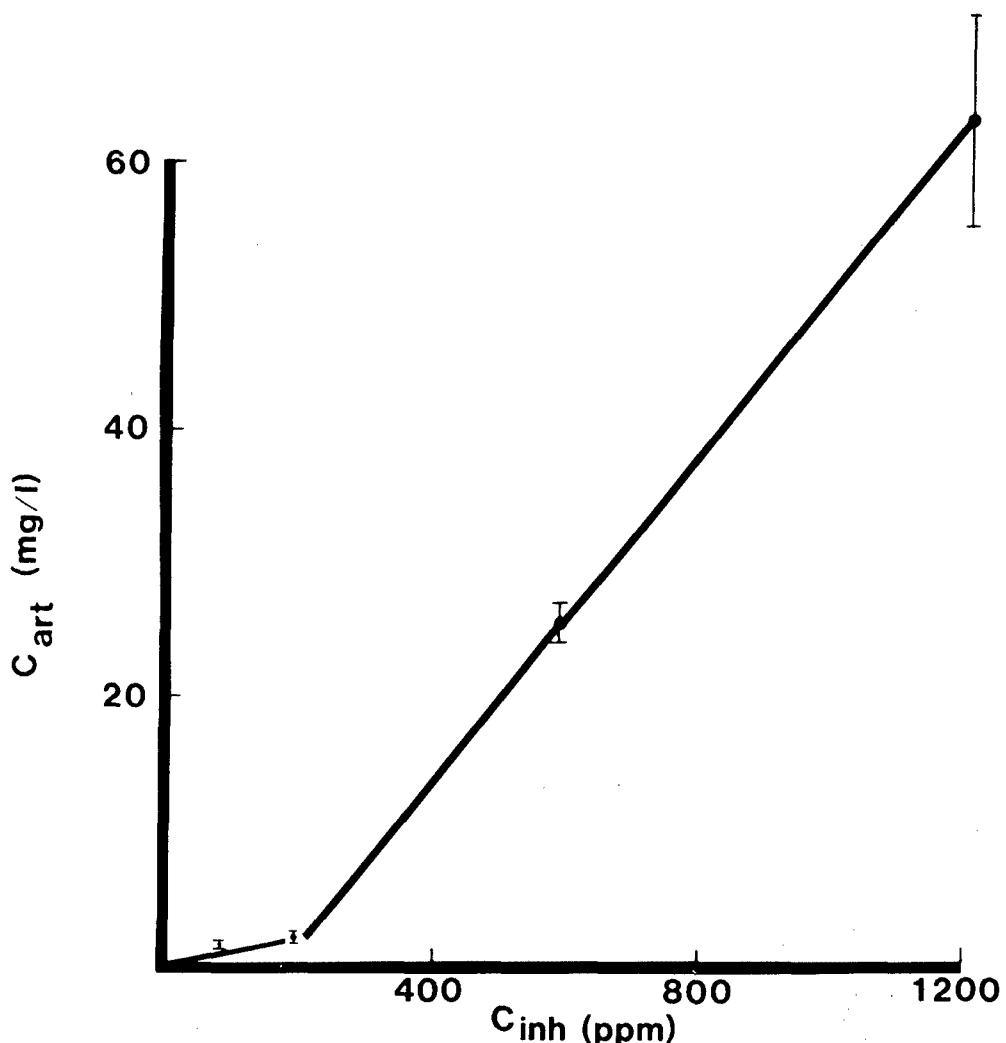


Figure 6. Relationship between steady-state blood concentration of styrene and its inhaled concentration. Time course profiles for styrene in rat blood were obtained during and after 6 hour exposures to various styrene concentrations by Ramsey and Young (1978). Actual values of the blood styrene concentration after 6 hours of exposure were kindly supplied by the authors for use in this Figure. Blood was obtained by decapitation.

Analysis of the compartmental model (Figure 1) also predicts a variable steady-state, blood/gas concentration ratio. At steady-state uptake into the blood compartment equals net loss from that compartment. This equality can be written in terms of compartmental clearances, as follows:

$$Cl_{12}(\text{air}) + Cl_{32}(\text{fat}) = Cl_{21}(\text{blood}) + Cl_{23}(\text{blood}) + Cl_m(\text{blood}) \quad (11)$$

Clearance subscripts, for instance xy , represent movement from compartment x to compartment y . Concentrations of vapor in each compartment are (air), (blood), and (fat). At steady-state Cl_{32} (fat) equals Cl_{21} (blood) and equation (11) reduces to:

$$Cl_{12}(\text{air}) = Cl_{21}(\text{blood}) + Cl_m(\text{blood}) \quad (12)$$

The steady-state blood/gas concentration ratio becomes:

$$S_{b,\text{eff}} = \frac{(\text{blood})}{(\text{air})} = \frac{Cl_{12}}{Cl_{21} + Cl_m} \quad (13)$$

When metabolic clearance, Cl_m , is much less than clearance by exhalation, Cl_{21} , the ratio approaches the blood/air partition coefficient ($S_b = Cl_{12}/Cl_{21}$). But when Cl_m is large, the proportionality constant will be less than S_b . Since Cl_m is concentration dependent for metabolized vapors, $S_{b,\text{eff}}$ is expected to vary with inhaled vapor concentration.

PRELIMINARY PHYSIOLOGICAL MODEL OF THE UPTAKE, DISTRIBUTION, METABOLISM AND ELIMINATION OF VAPORS

A promising approach to kinetic modeling of vapor inhalation is in expanded application of physiologically realistic descriptions of the test animal. In these models, compartments are defined as individual organs or groups of organs which are characterized by appropriate blood flows, organ volumes, and solubility coefficients for the test chemical. These physiologically-based descriptions have been widely used in anesthesiology research for many years. The mathematics involved in solving these models are complicated, but use of an electrical analog and digital solution of the equations germane to the analog have provided considerable insight into the pharmacokinetics of the inhalation condition (Mapleson, 1960; Fiserova-Bergerova et al., 1974, 1980). Another mathematical technique for describing the physiological system is to define movement of chemical throughout the system by a series of mass-balance differential equations (Bischoff and Brown, 1966). These equations are solved numerically to simulate expected kinetic behavior. In recent years this approach has been widely used to evaluate the pharmacokinetic behavior of anti-neoplastic drugs (Himmelstein and Lutz, 1979). These models are extremely versatile and it is likely that they will be eagerly and widely applied in inhalation toxicology in the near future (Andersen, 1981). One very appealing aspect of these physiological models is that they

allow ready extrapolation from test species to untested species based on the changing physiology and biochemistry from one species to the next (Dedrick, 1973).

For application to inhalation exposures, the lung must be described in some physiologically realistic fashion. Any complete description would include complex pulmonary dynamics such as the cyclic nature of respiration, the ventilation of tracheal-bronchial deadspace, and changing volumes of the terminal airspaces with respiration. A much simpler, first-approximation can be developed following the description of the ventilatory process used by Riggs (1970). For the present purposes, then, it is assumed that ventilation is continuous, that all material disappearing from the inspired air appears in arterial blood, and that equilibrium exists between alveolar air and arterial blood. The expression for arterial concentration, derived from the basic uptake relationship - equation (14), is simply:

$$C_{art} = S_b \frac{C_{ven} \dot{Q}_t + C_{inh} \dot{V}_{alv}}{S_b \dot{Q}_t + \dot{V}_{alv}} \quad (14)$$

where mixed venous blood concentration is determined from the effluent concentration from each organ/compartment of the model.

$$C_{ven} = \sum Q_i (C_i / S_{tissue:blood}) / \dot{Q}_t \quad (15)$$

The physiological model (Figure 2) contains three individual organs - lung, liver, and kidney - and three organ groups lumped together based on perfusion and solubility characteristics. They are designated other well-perfused organs (for example, brain, etc.), moderately well-perfused organs (muscle), and poorly perfused tissues (fat and marrow). The model is flow-limited; diffusion of soluble vapor from capillary blood into the intracellular milieu is assumed to be much faster than organ perfusion.

Mathematically, the mass balance differential equation for a metabolizing organ is:

$$\frac{V_i \frac{dC_i}{dt}}{dt} = \dot{Q}_i C_{art} - \dot{Q}_i (C_i / S_{tissue:blood}) - \frac{V_{max} (C_i / S_{tissue:blood})}{K_m + (C_i / S_{tissue:blood})} \quad (16)$$

Here V_i , Q_i , and C_i are the volume, blood flow, and soluble concentration in the i th organ. Effluent blood from the organ has a concentration given as the quotient of organ concentration divided by the organ:blood solubility coefficient. V_{max} and K_m are the kinetic constants of the enzyme system involved in solute metabolism. This relationship says simply that the change in mass of solute in the i th organ equals the rate of entry of solute with arterial blood minus the rate of removal by venous blood minus the rate of loss of solute within the organ by metabolism. This formulation assumes so-called venous equilibration behavior of the organs (Pang and Rowland, 1977), where organ blood is assumed to be in equilibrium with the effluent venous blood. Only the first two terms of equation (16) are important for a non-metabolizing organ.

To illustrate points in this discussion, two simulations were done based on the 5-compartment physiological model. Constants for the simulation were chosen to approximate those of a standard laboratory rat (Fiserova-Bergerova, 1976) and be representative of styrene exposures as described by Ramsey (1982) in this conference. Biochemical constants were selected such that metabolic clearance would be first-order at inhaled concentrations below 1 mg/L, but saturated at 4.8 mg/L (i.e., about 1200 ppm styrene). The blood/gas partition coefficient was set to 14.0; the fat/blood partition coefficient was 60; and tissue/blood solubility coefficients were 4.0, except for the moderately well perfused (muscle) tissue group where it was set to 1.0. V_{max} (liver) was 3 mg/hr; V_{max} (kidney) was 1 mg/hr; and K_m was 0.3 mg/L. The simulations (Figure 7a,b) represent 6-hour exposure to a constant concentration and the 18-hour period following cessation of exposure. The salient characteristics of the simulations are consistent with the predictions of the steady-state analysis and the observed pharmacokinetics of inhaled styrene in rats (Ramsey, 1982). At the lower concentration, $S_{b,eff}$ is small and equilibration is more rapid. At the higher concentration, the elimination phase is complex because the clearance pathway is saturated, and $S_{b,eff}$ approaches S_b . Because of extensive organ extraction, liver concentration is less than arterial concentration at the lower inhaled concentration and only at saturation does the liver/blood concentration ratio begin to approach 4.0.

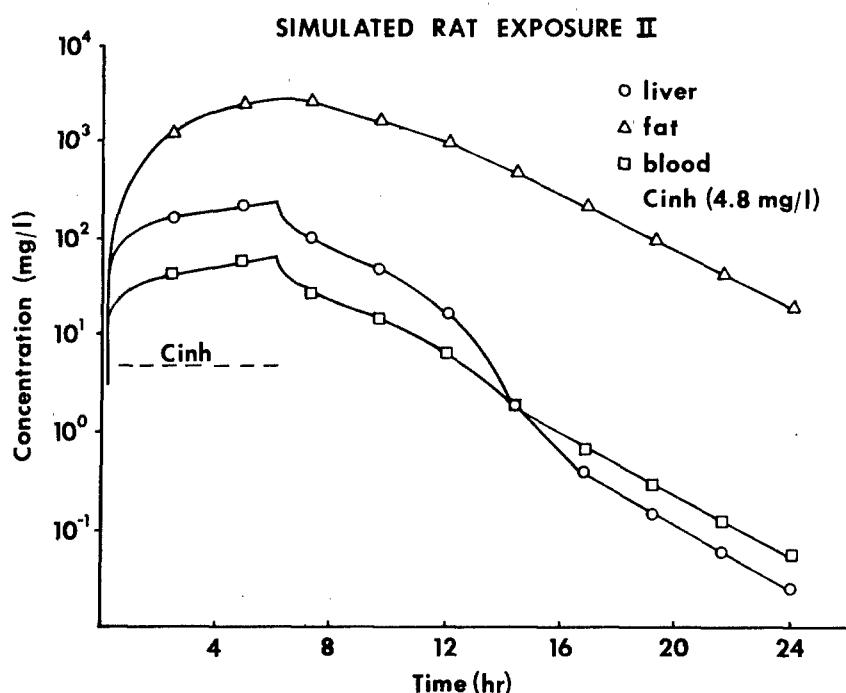
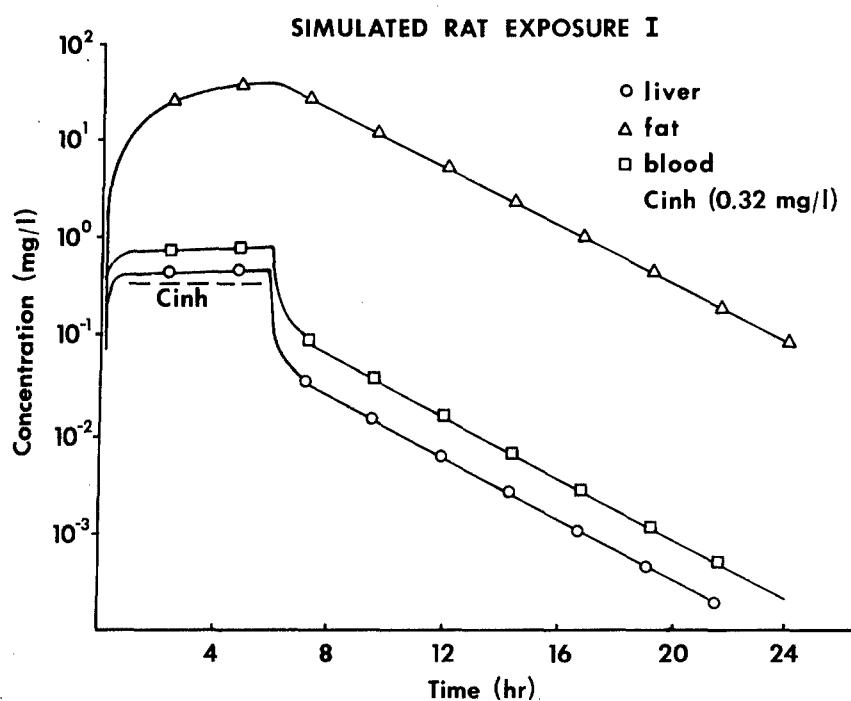


Figure 7a & b. Simulations showing the effect of exposure concentration of a lipid soluble, well-metabolized vapor on expected vapor concentration in the blood and tissues during and after a 6-hour exposure. Simulations were done based on the scheme in Figure 2. Flows and volumes for each compartment were: liver, 1.25 ℓ /hr and 0.02 ℓ ; kidney, 1.25 ℓ /hr and 0.002 ℓ ; other well perfused organs, 1.45 ℓ /hr and 0.010 ℓ ; moderately well-perfused tissues, 0.60 ℓ /hr and 0.16 ℓ ; & poorly perfused tissues, 0.45 ℓ /hr and 0.018 ℓ . Other constants were $\dot{Q}_t = 5.0 \ell$ /hr, $\dot{V}_{alv} = 5.0 \ell$ /hr, $V_{max, liver} = 3.0 \text{ mg/hr}$, $V_{max, kidney} = 1.0 \text{ mg/hr}$, and $K_m = 0.3 \text{ mg/}\ell$.

A. First-order clearance behavior prevails at low inhaled concentrations.

B. Saturation of metabolic clearance pathway prolongs uptake and leads to complex elimination after cessation of the 6-hour exposure. Simulations were done in collaboration with Dr. John Ramsey at the Dow Chemical Company, Midland, MI, using a commercially available software package-ACSL (Advanced Continuous Simulation Language).

Physiological models of this kind can be easily enlarged to include other aspects of kinetic behavior. Metabolites can be discharged to the blood, circulated to other organs, and eliminated via the kidney. Biliary excretion with enterohepatic recirculation can be incorporated. Exposures to mixed atmospheres can be modeled to include inhibitory interactions of one vapor on the metabolism of another. Indeed, whenever suitable differential equations can be written to describe the physiological processes, it should be possible to simulate expected kinetic behavior. These physiologically-based modeling strategies are extremely promising and their imminent, widespread application in inhalation toxicokinetic research should be extremely stimulating both in interpretation of existing pharmacokinetic data for inhaled vapors and in design of future experimentation in this area.

APPENDIX A

ABBREVIATIONS USED IN DESCRIBING PHYSIOLOGICAL MODELS OF THE UPTAKE, DISTRIBUTION, METABOLISM AND ELIMINATION OF INHALED GASES AND VAPORS

C_{blood}	Inhalant concentration in unspecified blood compartment (mg/l).
C_{art}	Inhalant concentration in arterial blood (mg/l).
C_{ven}	Inhalant concentration in mixed venous blood (mg/l).
C_{inh}	Inhalant concentration in inspired air (mg/l).
C_{alv}	Inhalant concentration in alveolar air after equilibration (mg/l).
C_{exh}	Inhalant concentration in exhaled air (mg/l).
C_i	Inhalant concentration in the i th organ of the physiological model (mg/l).
S_b	The blood:gas partition coefficient at 37°.
$S_{b,\text{eff}}$	The steady-state blood:gas concentration ratio. (Also called the effective steady-state partition coefficient.)
$S_{\text{tissue:blood}}$	The tissue solubility coefficient for a particular vapor: the ratio of solubility in the tissue to that in blood at 37°.

Amt_1 min	The amount of a metabolically inert gas taken up into an animal in the first minute of exposure (mg).
Amt_t	Amount taken up in the first t minutes of exposure (mg).
Amt_∞	Amount in the body at equilibrium (mg).
V_d	Volume of distribution (ℓ).
\dot{Q}_t	Total cardiac output (ℓ/hr).
\dot{V}_{alv}	Alveolar ventilation (ℓ/hr).
\dot{V}_t	Total ventilation (ℓ/hr).
$\dot{\mu}_{\text{s-s}}$	Uptake rate at steady-state (mg/hr).
$C_{1\text{xy}}$	Clearance of chemical from compartment x to compartment y (ℓ/hr).
E_t	Systemic extraction ratio.

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OPEN FORUM

DR. SLONIM (Air Force Aerospace Medical Research Laboratory): This question is addressed to both Dr. Ramsey and Dr. Andersen. I'm a little mixed up about this subject. When you're talking about styrene and you show a linear response and then it becomes nonlinear, what is your model, what is the significance of that? I think Dr. Andersen said that this reflects tissue saturation and if that's so in a concentration range of 200 to 1200 ppm that's a pretty long saturation phase. I'm more inclined to say it's probably limited by the rate at which you perfuse the chemical.

DR. ANDERSEN (Air Force Aerospace Medical Research Laboratory): The reason that the behavior of the uptake rate changes with styrene is a metabolic reason. With the metabolic constants set to the values that were used for that simulation and at low concentrations, about 1 milligram per liter in the arterial blood or 2 milligrams per liter, effectively all of the chemical that goes to the liver is removed. That is perfusion limited metabolism. But when the capacity for metabolism is exceeded which occurs somewhere above 2 milligrams per liter, then the system behaves quite differently. Those two curves show one case where the concentration going to the liver is much below the maximum that the liver can remove and another case where it's considerably above the maximum that can be removed by the liver. It is the saturation of metabolism that gives rise to the altered behavior. The exact natures of those elimination curves which I did not discuss in any length are really complex because they represent metabolic changes in removal of chemical from the blood that is stored in the fat and finally the rate at which it can get the chemical back from the fat into the blood to be metabolized. The exact nature of the last elimination phase which occurs from six hours onward in the last curve is complex and the nonlinearity in that system arises due to metabolism.

LT. COL. MacNAUGHTON (Air Force Aerospace Medical Research Laboratory): Whenever you have large models like we're operating here and you make certain assumptions about blood flow, organ weight size and so forth, you lock yourself into a model. Are there any published studies of a sensitivity analysis of how important it is to make critical assumptions about weight, blood flow, diffusion time, and so forth?

DR. BISCHOFF (University of Delaware): People have probably not yet made a truly formal sensitivity analysis in a mathematical sense for all the different equations, but just by trying different things out seems to show that these values, especially for the flows and the volumes, are obviously average values and of course wouldn't apply exactly to any single individual. The flows and the volumes aren't all that critical, which means that you can roughly scale them with body weight and it seems that the

models aren't overly sensitive to those parameters. If you look at the structure of the mathematic equations as actually the ratio of the flow to the volume, even though that's not the way they're written, the possible variations tend to cancel. The most critical parameters are the various clearances and the overall model results are, in fact, much more sensitive to those. That's why the basic tables that have been assembled for various animal species for the flows and the volumes seem to be able to be used more or less directly for a wide range of different drugs and situations, but the clearance rates are much more sensitive.

DR. RAMSEY (Dow Chemical Company): I agree with Dr. Bischoff. I think that the answer is going to be different for each compound. That may seem simplistic but a good example of that would be the data that you just saw on styrene. In this case the lipid solubility of that chemical means that the adipose or the fat compartment is really responsible for containing at any given time most of the styrene in the body and the parameters that are describing that compartment are more critical than some others. Because most of it is in one compartment, if you make a small change in that large mass it has a big effect on the rest of the system. It's a characteristic of the model and of the chemical which is being studied.

DR. HODGE (University of California, San Francisco): I'd like to ask Dr. Andersen if he could tell me about something that happened and occurs on that simulator rat number two. The blood curve sloped down linearly, the liver concentration started linearly then increased, went below the blood curve and after that was linear and parallel to the blood. I can understand why if the liver capacity to metabolize was relatively large, that would bring the curve for the liver down below that for the blood. Is that a reasonable explanation? But then, why didn't it continue to decrease more rapidly? Why, soon after the curve of liver concentration crossed the blood curve, did it run parallel and linearly? Is there something I've missed?

DR. ANDERSEN (Air Force Aerospace Medical Research Laboratory): Well, we'll go through it slowly and maybe I can explain the curves more clearly. In the first curve I show that at the low concentrations, the liver concentration and the blood concentration parallel each other throughout. And this is just a measure of the extent of metabolism not being extracted in the liver. At the higher concentration, when the exposure ceases, there is an initial small drop in the liver concentration but then much of the material is mobilized from the fat to maintain a circulating concentration coming to the liver that is slightly above the saturating concentration in the blood. While that concentration remains above this critical concentration, you maintain this difference. When the blood level gets close to this critical concentration of what can be metabolized, it starts to fall. And then it falls below liver concentration and then

after it has gone a sufficient distance below that, it is the equivalent of the initial very low concentration exposure where they've reached a parallel condition. Maybe somebody who has done some more simulations with models in which there is a storage depot involved could add something more.

DR. BISCHOFF (University of Delaware): What was the value of Km that you used in the simulation? Maybe that would help.

DR. ANDERSEN (Air Force Aerospace Medical Research Laboratory): The value is four milligrams per hour.

DR. BISCHOFF (University of Delaware): No, I asked for the units of concentration.

DR. ANDERSEN (Air Force Aerospace Medical Research Laboratory): I'm sorry, the Km is 0.3 milligrams per liter and the V_{MAX} is four milligrams per hour.

DR. COURI (Ohio State University School of Medicine): The point size of the animal is a factor but shouldn't we really be talking about the size of the site of action? Or the body size in relationship to the site of action? I've heard two or three definitions here of pharmacokinetics meaning the intensity, meaning the amount of chemical that remains in the organism or in the body and by implication means at some organ. But really it's not even an organ in a sense, is it? An amine has had a specific locus in the organ where this action takes place so the best modeling we can get to really can't relate to the amount of chemical that acts at a very specific locus. I'd like at least one of the pharmacokinetic modelers to suggest a manner in which we can reach a little more of the specificity of what we're alluding to pharmacokinetics, specifically how one examines what you can predict with respect to chemicals acting on systems? We talk about modeling and the model means nothing until you do an experiment and then generally the experiment can stand by itself. I don't think we need a model for the experiment. If we've got to do the experiment, then why do we need a model? The model is not verified until an experiment is done: when will we be able to do the model and not do the experiment? Is that possible?

DR. BISCHOFF (University of Delaware): Maybe the best answer is an historical one and that is in pre-Newtonian days, people did lots of experiments like Gallileo dropping balls off of leaning towers and Kepler observing planets and so on. I'm sure that these same statements would have been made then, that they had all of these strange observations that couldn't be understood. Obviously at this point in time, using Newton's laws which are so much simpler we don't have to take all those observations anymore because we know that we could in fact predict which rocket is going to hit which planet.

DR. COURI: When will the model give us predictability without having to do the biological experiment? I agree with you that I would rather not do the experiment.

DR. BISCHOFF (University of Delaware): Well, what I was trying to say was that that was obviously a much simpler system and we have rather good theories, basic theories and obviously there we are able to truly predict things and the astronomers can predict what will happen 50 years from now and I think we agree they're probably going to be correct. Now, when it comes to chemical things, we obviously can't do as well and when it comes to biological things, we can be even less skillfull in making true prediction. I think that probably within our lifetimes we won't ever be able to do this in an a priori way. On the other hand, the purpose of a model perhaps in a more practical sense is that it never eliminates experiments but it might reduce the need for as large a number of experiments if you can verify parts of the model and feel that you are on the right track with your model without having to do an exhaustive series of experiments. It might also make what experiments are done much more effective by forcing us to answer various specific questions about what is happening and what are the alternatives. I completely agree with you that if the only purpose of a model is to write equations that will fit the same data that we have anyhow, there is no need for the equation. We hope that these physiologically based models can use data that have been measured in a more general sense to partially predict what is going to happen in the disposition. Now, your other point is that the pharmacokinetics is not the end answer and what we really want to know is the drug effects, and pharmacokinetics is only part of the answer there. What we really want is the site of action level. So I guess once again my overall answer is that we have to start somewhere just as scientists did in the 1500's and 1600's when they didn't have any neat theories; we can't do all this yet, we can do some of the easier things and perhaps get those out of the way and then spend more of our efforts on the hard things that we can't do as well such as pharmacodynamics, namely, the drug action.

DR. RAMSEY (Dow Chemical Company): Basically I agree that the ultimate goal is the same, that what we're really trying to get at is the concentration of the ultimate toxicant at that sensitive locus. I feel that pharmacokinetic modeling is a step in that direction. I also feel that that goal is a long way away because we really accept that as the criterion for success and when we get there, we can literally shut down the animal rooms and buy computers as far as toxicology is concerned and I wish we could do that. But it's going to be a long time.

SESSION V

ENVIRONMENTAL EFFECTS

Chairman

**Maj. James M. Livingston
Chief, Environmental Quality Branch
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**ASSESSING THE HAZARD OF CHEMICALS TO AQUATIC LIFE:
A PERSPECTIVE ON THE STATE-OF-THE-ART**

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INTRODUCTION

Aquatic ecosystems have historically been used for the convenient disposal of human and industrial wastes. The inevitable result of population growth, industrialization, and urbanization has increased water pollution. The degradation of water quality in many estuaries, rivers, and lakes made it obvious that the capacity of waterways to assimilate wastes was indeed limited. The significant growth of the chemical industry during the past century and increasingly sophisticated analytical chemistry techniques that made possible the detection of trace quantities of numerous chemicals heretofore unknown in natural waters served to further stimulate interest in the protection of our aquatic resources.

In recent years much attention has been directed toward the assessment of the hazards associated with the manufacture, distribution, use, and disposal of chemicals. Many countries are currently evolving legislation to regulate the introduction of new chemicals and to provide for the review of existing chemical compounds (e.g. the U.S. Toxic Substances Control Act, 1976; European Economic Community, Directive 79/831/EEC). It has been estimated that there are over 4,000,000 distinct chemical entities, that approximately 63,000 of these chemicals are in common use, and that several hundred new chemicals annually have the potential for environmental release (1,2). The necessity to formalize procedures for assessing chemical hazards and deciding whether a given chemical presents a reasonable or unreasonable risk to human health or the environment is apparent. Numerous plans for this purpose have been advanced in recent years (e.g. 3-11). It is not, however, the objective of this discussion to review any of these published plans in any detail. Rather, the purposes of this review are to identify and discuss the basic principles involved in assessing the hazard of chemical substances to aquatic life and to describe the conceptual framework that forms the basis of these specific assessment procedures. It is

a further intention to provide a perspective on some future research needs in the areas of aquatic toxicology and chemical hazard assessment.

CURRENT CONCEPTS OF ASSESSING THE HAZARD OF CHEMICALS TO AQUATIC LIFE

In dealing with this subject it is important to have a thorough understanding of the basic terminology involved. For the purpose of this discussion the terms hazard and risk may be used interchangeably. Both terms are synonyms for the word danger and each implies an element of chance that an adverse effect will occur under a given set of conditions. The hazard assessment of a chemical has been defined as a prediction of the magnitude and length of duration of chemical concentrations occurring in the various segments of the environment resulting from the use of the compound compared with the concentrations of the chemical in food, water, or air that are known to be harmful to representative species, populations, and ecosystems (12). Such a definition clearly indicates the kind of information that is essential for assessing the hazards of a chemical to aquatic life (Figure 1).

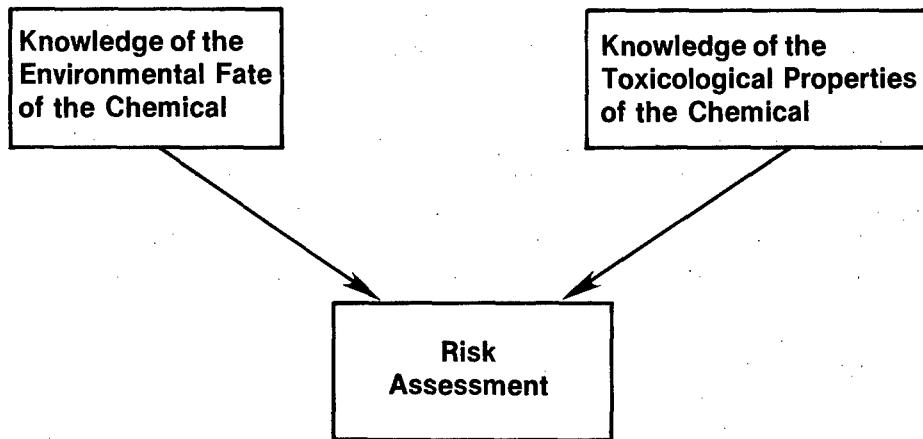


Figure 1. Information needs for the risk assessment process.

Toxicity is defined as the potential of the chemical to induce unwanted or deleterious effects in biological systems. Toxicity is an inherent, unalterable property of the chemical and is a function of the exposure concentration, or dose, and the nature of the biological system exposed. Toxicity and hazard are not synonymous terms. It is unfortunate that the redundant terms "toxic substance" and "toxic chemical" enjoy such widespread use, for scientists recognize that all chemicals have the potential to

be toxic to some biological system under appropriate exposure conditions. It is of course essential to learn about the inherent toxicity patterns associated with a chemical in order to complete the risk assessment.

It is equally important to learn about the nature and degree of exposures that might result from the manufacture, use, or disposal of the chemical. This requires a thorough understanding of the environmental fate of the chemical. The fate of a chemical in the aquatic environment may be thought of as the concentrations resulting from all point and non-point source inputs, as modified by the chemical, physical, and biological transport and transformation processes that are active in the environment.

Safety may be defined as a value judgment of the acceptability of risk. A chemical is regarded as "safe" if its associated risks are judged to be acceptable. Such a definition implies that two different activities are required for determining safety. First, a risk assessment is necessary to evaluate potential harm to aquatic organisms. Second, a value judgment regarding the acceptability of that risk must be made.

THE RISK ASSESSMENT PROCESS

Basically in any risk assessment process, two lines of scientific investigation seek to relate observed biological effects to predict exposure concentrations (Figure 2) (5). Theoretically, there exists a concentration of a particular chemical substance that can be determined to have no adverse effects on survival, growth, or reproduction of representative aquatic life. This concentration is typically referred to as the no observed effect concentration (NOEC) and is usually determined from laboratory toxicity testing of "representative" aquatic organisms. Similarly, there exists a highest predicted environmental concentration (PEC) that will result from the normal anticipated use of the chemical during manufacture, transport, and consumer use. It thus becomes basic to the risk assessment procedure to accurately measure or estimate these two concentrations so that a relative comparison of the difference between the known biological effects and the environmental concentrations can be made. Figure 2 represents the two concentrations as parallel lines and demonstrates that increasingly more accurate and statistically reliable estimates of these concentrations will result from a sequential series of tests completed in time. Several modifications of this conceptual representation of the risk assessment process have been suggested in order to more accurately reflect the nature of both biological variability and fluctuating exposure concentrations (13,14). In all cases, however, the process of determining and comparing the fate and effects of chemicals released to the environment leads to risk assessments which form the bases for decisions about the acceptability of that risk.

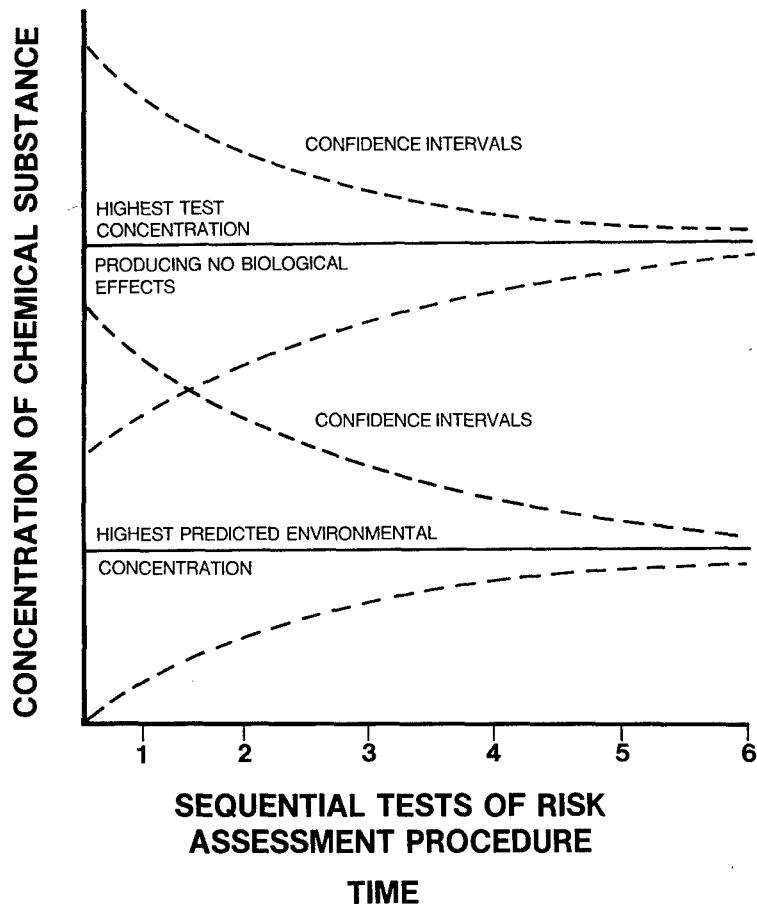


Figure 2. Diagrammatic representation of the risk assessment process demonstrating the increasingly narrow confidence limits for predicted environmental concentration and estimates of no adverse biological effects concentration. From (5).

In the early phases of the risk assessment process, estimates are made of biological effects and environmental concentrations; however, the wide confidence intervals that overlap indicate that additional data are needed to determine if the two concentrations are statistically different. As the risk assessment process proceeds, increasingly more accurate estimates of fate and effects can

be made to the point where it becomes possible to state with a high degree of confidence that environmental concentrations and biological effect concentrations are indeed different. It then becomes a matter of judgment to determine just how far into the assessment process the investigator should proceed to further narrow confidence around fate and effect concentrations.

HAZARD ASSESSMENT PROGRAMS

Many factors and their appropriate combination must be considered in carrying out an effective yet efficient hazard assessment program. A generalized program is presented as a flow chart consisting of a number of distinct phases (Figure 3). Each phase is designed to accomplish certain scientific purposes and all of them are interrelated. In each phase there are provisions for acquiring and organizing information and for making certain kinds of decisions. Flexibility is provided so that the key issues for any particular chemical are addressed. Generally, experimentation flows from relatively simple tests to increasingly complicated ones. The amounts and kinds of information needed in each phase are largely independent of the amounts needed in other phases even though the phases are closely integrated. The flow chart presents a "flow of thinking" as contrasted with the more familiar steps or tiers which constitute a "flow of activities". After considering the physical and chemical characteristics of the material and potential routes of exposure, important questions are identified and tests are then selected to answer those questions. To work properly and efficiently, the assessment program in practice must contain an element of specific decision criteria or pass/fail options to provide direction in decisions concerning future use or scope and priority of additional data required in the overall hazard evaluation. Without these decision criteria imposed throughout the evaluation, the entire process degenerates to a checklist of required testing/data having no mechanism to indicate when the process has been successfully or satisfactorily completed. As more information is developed, a point is reached at which decisions can be made regarding the safety of the substance in question. Sometimes the decision is that the substance presents too high a risk. Other times it can be decided there is an acceptable risk or that more work needs to be carried out. Scientific judgment is used in deciding which tests to do and when sufficient testing has been conducted. The collection of decisions is then integrated into one overall decision concerning the acceptability of the material in terms of both human and environmental health.

FLOW CHART FOR SAFETY ASSESSMENT

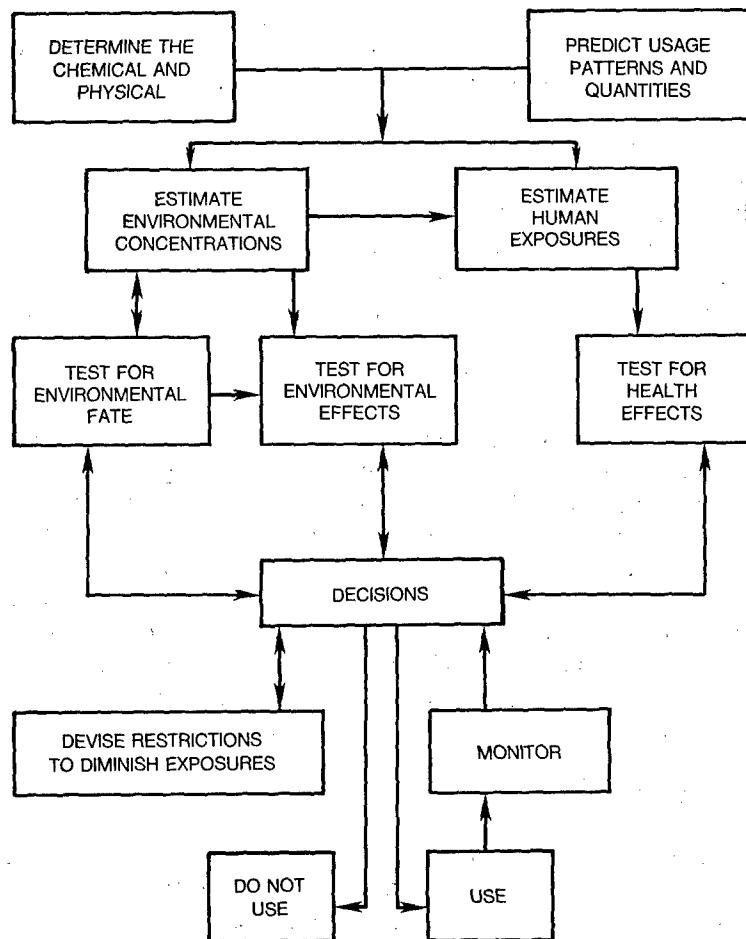


Figure 3. Flow chart for the generalized safety assessment process. From Beck et al., 1981.

The generalized outline provided here, having been derived from our experience of recent years, is only intended to provide insight into the nature of hazard assessment programs. No made-in-advance outline can be broad enough to accommodate all kinds of chemicals for all kinds of uses or be flexible enough to accommodate the developments in safety testing that are continually emerging.

In accordance with Figure 3, the process of making and reviewing a safety judgment can be analyzed as consisting of ten individual components. The sequence of the process is split into two paths, one for human safety and one for environmental safety, which come together again at the point of decision making. Each of the components is described briefly below.

1. Physical/Chemical Properties:

The evaluation starts with a consideration of the properties of the material, ascertained from the literature if possible, determined in the laboratory if necessary. Estimates may suffice in the early stages, but more precise determinations will generally be needed later. This is the time for initial development of the analytical methodology that will be needed.

2. Usage Patterns:

How the material will be used and in what quantities must be considered, since usage patterns (along with such related matters as manufacturing, shipping, and disposal) determine the routes and amounts of both human and environmental exposure.

3. Environmental Concentrations:

From anticipated usage patterns, the concentrations that can be expected in various environmental compartments are predicted. These estimates can be helpful in projecting human exposures, but their chief value is in suggesting how extensively the materials should be tested for environmental fate. Then, from the results of the environmental fate tests, more refined predictions of environmental concentrations can be made. These estimates are necessary both for predicting human exposures and for interpreting the results of the tests that will be done for evaluating environmental effects.

4. Environmental Fate:

Tests for environmental fate reveal what happens to the material after it is released into the environment. This kind of information is necessary for making the refined estimates of environmental concentration referred to above, and this is one of the factors influencing the estimate of human exposure.

5. Human Exposure:

Estimates of human exposure can be made from information about manufacturing, transport, and usage patterns and the estimates of environmental concentration. A number of estimates are needed to cover such diverse situations as exposure of factory workers, intentional and accidental exposures of consumers, and incidental exposures of humans from drinking water or air. Exposure may be oral, respiratory, ocular, or dermal routes. These estimates of exposure are of value in determining which health effects tests should be carried out, and are essential in evaluating the results of those tests.

6. Health Effects:

Tests for health effects are concerned with the possible effects of the material on human health, but most of the tests are carried out with laboratory animals. From information about the kinds of effects produced in these animals, and the dosages of material necessary to produce them, it is possible to estimate the dosages of material that would be safe for humans.

7. Environmental Effects:

Tests for environmental effects indicate whether the concentrations expected may cause harm to the environment, particularly to the living creatures in it, the kinds of injury that may occur, and the species most likely to be affected.

8. Decision Making:

The first step in decision making is to compare the dosages of a material that are supposed to cause harm to humans or the environment with the dosages that will result from using the material. From this comparison, it is possible to assess the risk of causing harmful effects. Finally, the decision is made whether the risks are societally acceptable or not. It is not necessary to do all the tests that are possible before making this decision; there must be provisions at many points in the process for deciding that no further testing is necessary.

9. Monitoring:

If the decision is to use the material, field monitoring may be needed to determine whether the resulting environmental concentrations correspond to those that were predicted from laboratory testing. Medical follow-up of consumer comments and surveillance of employee health can also be useful ways to check on the correctness of the decision to proceed.

10. Restrictions on Use:

If any of the risks associated with using the material as originally planned are judged unacceptable, it may be possible to devise restrictions on the use of the material that would diminish the anticipated exposure and therefore lower the risk. The restrictions might be of many sorts, ranging from warning labels to the construction of containment dikes around storage tanks. Once such restrictions have been devised, it will then be necessary to go through parts of the decision making process again to see whether the risk is now acceptable.

FUTURE RESEARCH NEEDS

A number of recent publications have discussed the history, evolution, current trends, and future research needs in aquatic toxicology and hazard assessment (12,14-24). Consideration of the priority of these needs is obviously influenced by the individual's viewpoint (e.g. regulatory, academic, industrial, legal, etc.) and the reader is referred to the literature cited in order to obtain an overall summary of current thinking. What follows is the author's perspective on some of the most critical research needs.

BIOAVAILABILITY

Current laboratory toxicity test methods typically specify that testing be conducted in well characterized, high-quality dilution water. Extrapolation of these data to real world situations is often difficult, misleading, and can lead to unnecessarily lenient or restrictive regulations. Do our laboratory studies accurately reflect conditions in nature where a variety of chemical, physical, and biological interactions may detoxify, degrade, photolyze, hydrolyze, and adsorb the chemical and/or otherwise regulate its availability to aquatic life? Do tests conducted at a constant exposure concentration provide useful information for assessing the hazard of periodic or widely fluctuating exposures in natural systems? Improved procedures are needed which consider the effects of such variables on the chemical's environmental fate. Cairns (25) has described the need for this "environmental realism" in test methods. An evaluation of the bioavailability of a chemical in natural waters must be considered as part of the hazard assessment.

DETERMINING THE ECOLOGICAL SIGNIFICANCE OF LABORATORY DERIVED DATA

Can laboratory data on fate and effects be used to predict the ecological impact of a chemical released into the aquatic environment? The question is appropriate to all aspects of applied aquatic toxicology (e.g. environmental impact assessment, hazard assessment of new expanded-use chemicals, development of water quality criteria/standards, etc.) and covers a multitude of other more specific research needs.

- Utility/limitations of single species tests - It is a key assumption (often unstated) of most hazard assessment programs that the results of single species toxicity tests can be used to protect ecosystems. The validity of such an assumption is certainly less than well established, or even tested (24,25).

- Comparative toxicology - Do studies with "representative" test organisms traditionally used in toxicity tests provide adequate information to characterize the variability in species sensitivity? Do we in fact frequently test an appropriate array of species? The development of sound theory in the area of comparative toxicology with regard to relationships between phylogenetic groups could enable us to begin to practice predictive toxicology at the organisms and species level (26). Research programs are needed to better understand the anatomy, pathology, histology, histochemistry, physiology, biochemistry, genetics, nutritional requirements, and life history of our test species.
- Modes of action/toxic mechanisms - To date, aquatic toxicology has been preoccupied with measuring effects such as mortality or reproductive success. Basic research in mechanisms of toxicity in aquatic organisms has been limited. Information on toxic mechanisms at the organ, cellular, or molecular level would enhance our understanding of structure-activity relationships. Information so derived has broad implications for human health considerations as well, and aquatic organisms are being widely utilized as model systems in studies of teratogenicity, mutagenicity, carcinogenicity, and in other fields of biomedical research (27).
- Relevance of test endpoints - The relationship of end-points typically measured in laboratory toxicity tests (e.g. lethality, alteration in growth rate, reproductive success, behavior, etc.) and effects on population dynamics under field conditions is not well understood. The distinction between statistical significance (a function test design) and biological/ecological significance is often overlooked. What is the relationship between a 25% reduction in total young produced in a laboratory population of Daphnia magna and the effect on indigenous zooplankton populations in nature? Does a 50% reduction in the growth rate of a unicellular culture have any real-world ecological counterpart? Research programs such as the "Shayler Run" study (28) can be useful for providing information on whether the results of laboratory tests can be correlated to the results obtained from field tests with the same chemical.

ASSESSING IMPACT ON AQUATIC ECOSYSTEM FUNCTION

Research in aquatic toxicology has historically been conducted at the organism and population level of organization. Relatively few test systems have been developed to specifically assess the effects of chemicals on ecological systems, interspecies interactions, community dynamics, or ecosystem properties. We need to move away from strict reliance on single species tests to those

procedures wherein the interaction of different species and effects on functional processes can be evaluated (24). The adequacy of protection afforded by current assessment techniques may be judged differently by different segments of society. The public may judge protection on the basis of community features (i.e. a healthy sport fishery) while the scientists/ecologist may judge adequacy on the basis of community functions (29). Concomitant with advances in our understanding of the structure and function of aquatic ecosystems, we must develop methodologies to assess the impact of chemicals on those processes which regulate ecosystem function such as carbon fixation, detritus processing, biomass production, nutrient cycling, and energy flow (16,24,25,30).

SUMMARY

A conceptual framework has been developed for assessing the hazard of chemical substances to aquatic life. A hazard assessment is based on the relationship between the environmental exposure concentration of the material and the concentration showing some adverse biological effect. Any program or guideline for environmental safety testing must contain a strong element of flexibility with respect to testing priority and test selection. Specific questions associated with each material should be identified during early comprehensive screening tests which subsequently lead to individualized testing and experiments designed to position risks against intended use.

Satisfactory approaches and methods are available to predict the environmental fate of chemicals introduced into the environment, and test methods exist for determining adverse biological effects. However, a need exists for advancements in both of these critical components of hazard assessment. Although hazard assessment procedures are in the developmental stages, it is evident that the respective approaches have reasonable philosophical foundations and are functional. However, they need to be utilized and tested with data developed on chemical substances for which longer-term use has defined the safety to aquatic life. After such testing, it is highly probable that refinements may be needed in the procedures. In addition, the criteria used in the procedures to make decisions on the need for additional testing must themselves be critically scrutinized to firmly establish their scientific credibility. Such scrutiny can only come from repeated applications of these assessment procedures to a wide variety of test substances that have the potential to reach surface waters.

It is unreasonable to expect that any preconceived testing program for comprehensive safety assessment could contain sufficient foresight to anticipate each issue of testing that could

potentially arise during the evaluation. An extreme but impractical alternative is to specify all tests for all chemicals; however, it is believed that the more reasonable and scientifically valid approach outlined in this review, coupled with the necessary flexibility in test selection presents the best option for efficient decision making.

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BEHAVIOR OF HYDROCARBON FUELS IN AQUATIC SYSTEMS*

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Five high density fuels (RJ-4, RJ-5, RJ-6, JP-9, and JP-10) and three distillate fuels (JP-4, JP-5, and JP-8) were analyzed by capillary column gas chromatography (GC). The major components of the distillate fuels were identified by gas chromatography-mass spectrometry (GC-MS). The molecular weight of the isomers of the high density fuel components were also determined, but the structures of only a few components were assigned by comparing the GC retention times with authentic samples.

The concentration and identity of the major water-soluble fuel components were also determined. The major aromatic components were benzene, the xylenes, 1,2,4-trimethylbenzene, naphthalene, and the methylnaphthalenes.

The solubility of the high density fuel components was less than $0.02 \text{ mg liter}^{-1}$ except for exo-tetrahydrodi(cyclopentadiene) which was less than $0.1 \text{ mg liter}^{-1}$. A correlation between the water solubility of the fuel component(s) and the fuel-water partition coefficient, K_{fw} , was found, where $K_{fw} = 0.799 \log S + 1.664$ for JP-4, JP-5, and JP-8 at 20°C in deionized water at a fuel:water ratio of 1:1000.

The volatilization rates of the water-soluble components of JP-4, JP-8, and JP-9 were measured by preparing solutions of the fuel components in water, stirring at three stirring rates, and measuring the rate of decrease of the concentration of each component by GC as a function of time. The ratio of the component volatilization rate constant to the oxygen reaeration rate constant

* This paper presented by Lt. Col. Michael G. MacNaughton, USAF, BSC, Deputy Director of Air Force Aerospace Medical Research Laboratory/Toxic Hazards Division, Wright-Patterson Air Force Base, Ohio 45433.

was measured. The average value of this ratio for the alkanes and substituted benzene components was 0.52 ± 0.09 , which means that liquid phase mass transport resistance determines the volatilization rate. The estimated half-lives in the environment were 7 days in ponds, 1.5 days in rivers, and 6 days in lakes. The volatilization rates of naphthalenes, the methylnaphthalenes, and perhaps the tetra-substituted benzene derivatives were somewhat slower, suggesting that both gas and liquid phase mass transport resistance determine their volatilization rates.

The water-soluble components of JP-4 were photolysed for 21 days in sunlight in deionized water, synthetic seawater, and water from a local pond. The alkanes, benzene, toluene, the mono- and di-substituted benzenes were stable. The tri-substituted and higher benzenes and the naphthalenes were transformed at rates that were competitive with their volatilization rates.

The distribution of the fuel components was estimated, using the method recently proposed by Mackay and Patterson. The alkanes should partition almost entirely into the atmosphere, the monoaromatics should be in both the air and water, and the naphthalene should partition into the water and sediment phases. Adsorption of the alkanes and monoaromatics should not be a major environmental fate. Although quantitative rate estimates were not made, the primary environmental fate of many of the alkane and monoaromatics should be transport into the atmosphere where photolysis should be rapid.

It is recommended that the rate of dissolution and evaporation of the pure fuels be studied in detail because these processes may be the rate-limiting transport processes.

DETERMINATION OF THE WATER-SOLUBLE FRACTION

The partitioning of JP-4, JP-8, and JP-9 between the bulk fuel and deionized water or synthetic seawater was measured at several fuel:water ratios. A major problem in these studies was to be sure that no droplets of the fuel were present in the aqueous phase equilibration. Another problem was to minimize the rapid volatilization losses of the dissolved fuel components that may occur during handling of the aqueous solutions.

A solution of the water-soluble fuel component fraction was prepared at a fuel:water ratio of 1:1000 by injecting 100 μl of fuel from a syringe into 100 ml of water contained in 150-ml centrifuge tubes to disperse the fuel as tiny droplets in the water phase. The tube was sealed with a Teflon-lined screw cap and gently mixed by rotation for 48 hours in a constant temperature bath. Following

equilibration the sample tubes were centrifuged for 30 minutes at 5000 rpm, which produced a relative centrifugal force of approximately 1500 g at the solution surface and 3500 g at the bottom of the tube. Then the fuel was siphoned from the water's surface. A pipet was used to remove 5-ml aliquots of the remaining solution which were extracted with CS₂ containing a known amount of n-octadecane as an internal standard. Since the density of RJ-6 was slightly greater than the water in which it was equilibrated, centrifugation caused the fuel to coalesce at the bottom of the vessel; the other fuels floated on the water surface.

The major components in the fuels were identified by GC and GC-MS and quantitated by GC. All components of the high density fuels except one isomer of JP-10 have very low solubilities. Minor aromatic impurities in these fuels were easily detected in the water-soluble fraction. The water-solvent blanks were carefully checked to assure that these aromatic compounds were indeed in the fuels.

As expected, the solubilities were generally lower in seawater than in deionized water at the same temperature. At a fuel:water ratio of 1:1000, the type of water has little effect on the partitioning. However, changing the fuel:water ratio from 1:10 to 1:10,000 significantly reduced the concentration of the water-soluble fractions of JP-4 and JP-8 and of the minor components of JP-9.

VOLATILIZATION FROM WATER

The volatilization rates of the major water-soluble fuel components in JP-4, JP-8, and JP-9 were measured from water equilibrated with the fuel at a 1:1000 fuel:water ratio, using the method described by Smith et al.*. Fuel-saturated water (500 ml) was stirred in a 600 ml beaker. During each experiment 5-ml samples were removed at appropriate time intervals and immediately extracted with CS₂ containing n-octadecane as an internal standard. The extracts were then analyzed by capillary column GC. The volatilization rate constants were calculated for each compound.

Following collection of the last sample, N₂ was introduced to purge the solution of oxygen. Without changing the stirring rate, the rate of solution reaeration was monitored with a dissolved oxygen analyzer. The oxygen reaeration rate constant was then calculated.

* Smith, J. H., D. C. Bomberger, and D. L. Haynes (1980), Environ. Sci. Tech., 14(11):1332.

The values for k_v^C/k_v^O are summarized in Tables 4 and 5. All the water-soluble fuel components are high volatility compounds ($k_v^C/k_v^O \approx 0.52$) except for naphthalene and its derivatives. The concentration of the norbornadiene dimers (RJ-5 isomers) in water was so low that volatilization rate measurements were not possible.

PHOTOLYSIS

The influence of exposure to direct sunlight on the water-soluble components of JP-4 was studied in deionized water, a natural fresh water, and a natural salt water. Before equilibration with the fuel, the two natural waters were filtered and centrifuged to remove suspended particles and microbes. Aqueous solutions of JP-4 were prepared from 1:1000 fuel:water mixtures as previously described. The water solutions were then transferred by pipet to quartz photolysis tubes, and the tubes were sealed.

The photolysis tubes were placed on the roof on a rack along with dark control samples. After 7, 14, and 21 days of light exposure, one light exposed and one dark control tube for each of the three waters was removed for sampling. A 5-ml sample was removed from each tube and immediately extracted with CS_2 containing n-octadecane as an internal standard. The extracted samples were analyzed by capillary column GC.

The data, which are summarized in Table 6, suggest that experimental errors were greater than the disappearance of any of the water-soluble components of JP-4. The other distillate fuels are expected to give similar results. The high density fuels do not absorb ultraviolet light in the solar region and therefore should not be photochemically reactive.

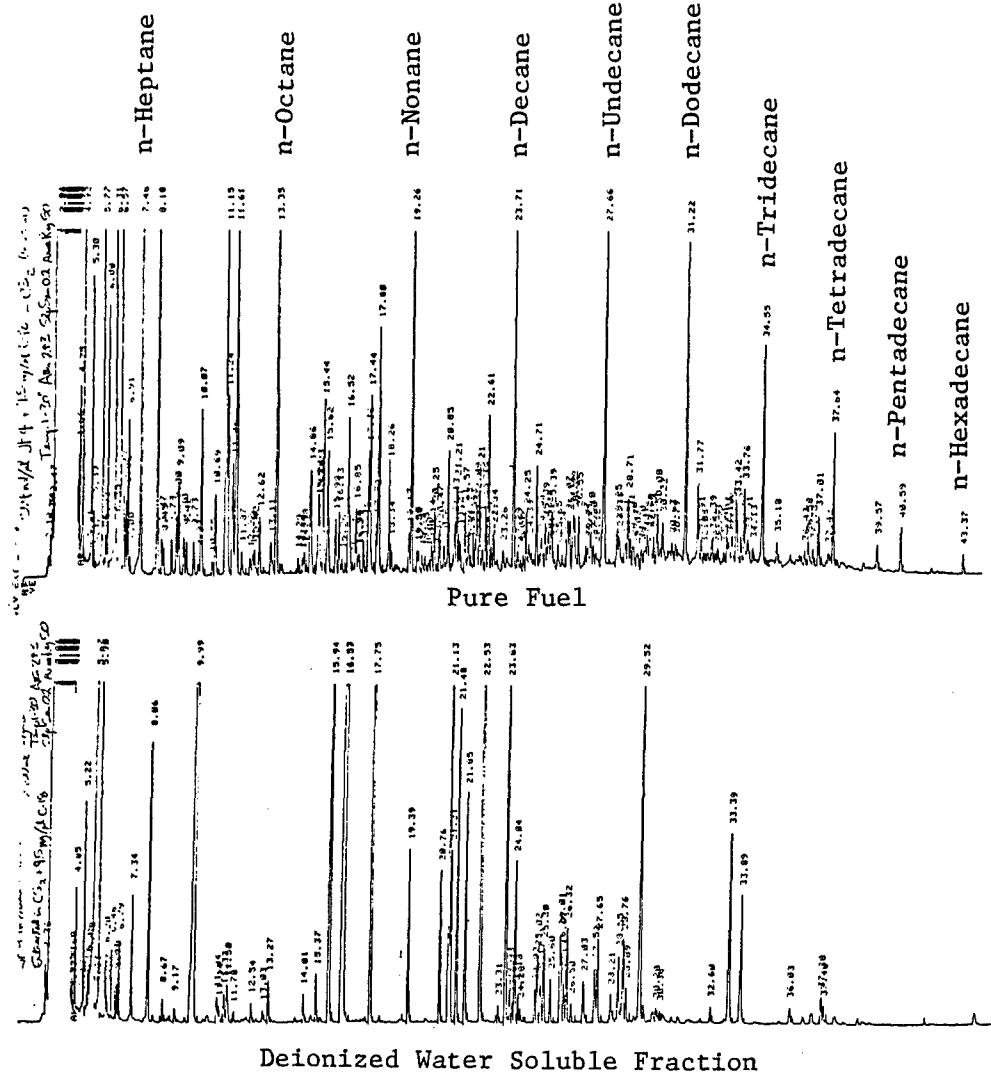


Figure 1. GC traces of JP-4 in CS₂.

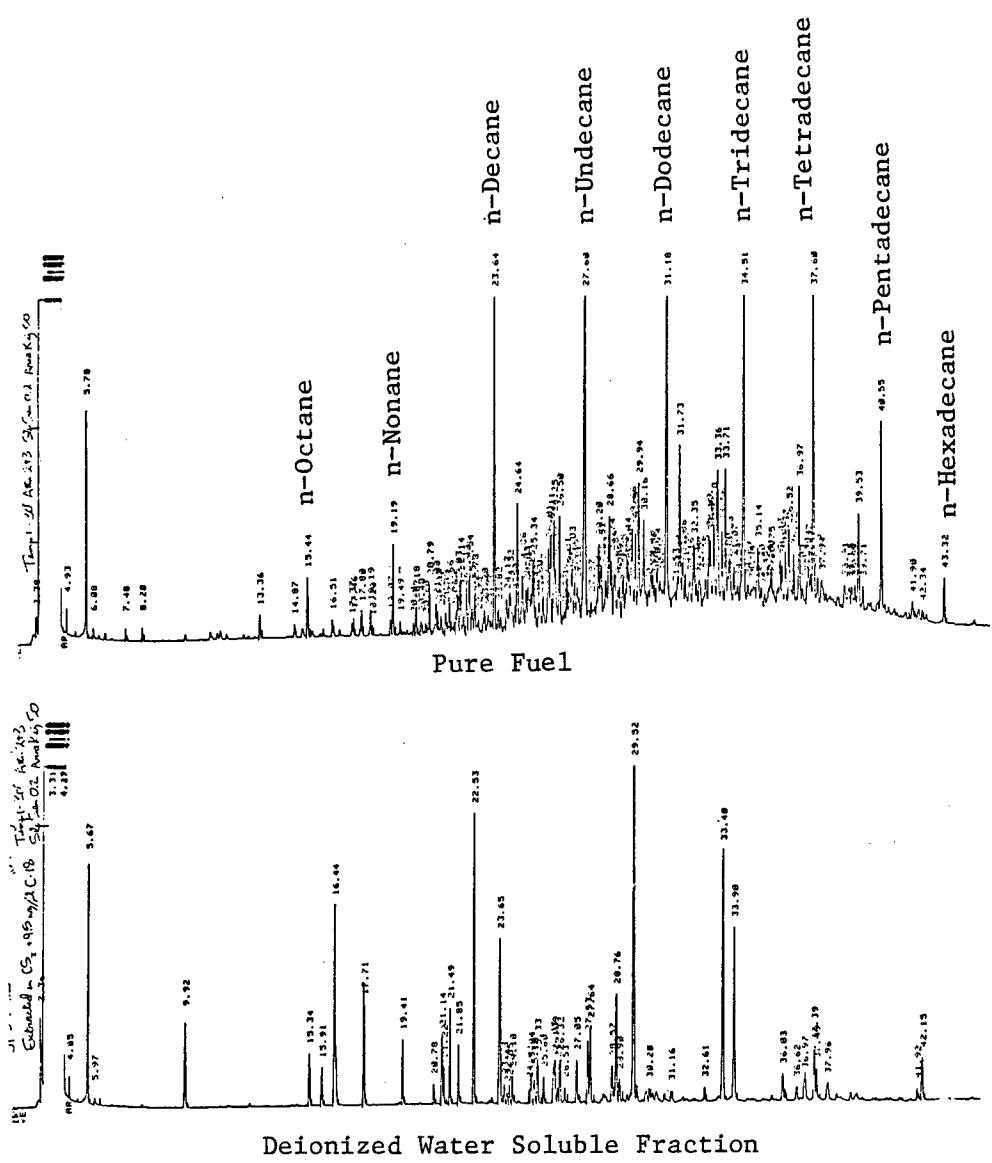


Figure 2. GC traces of JP-5 in CS₂.

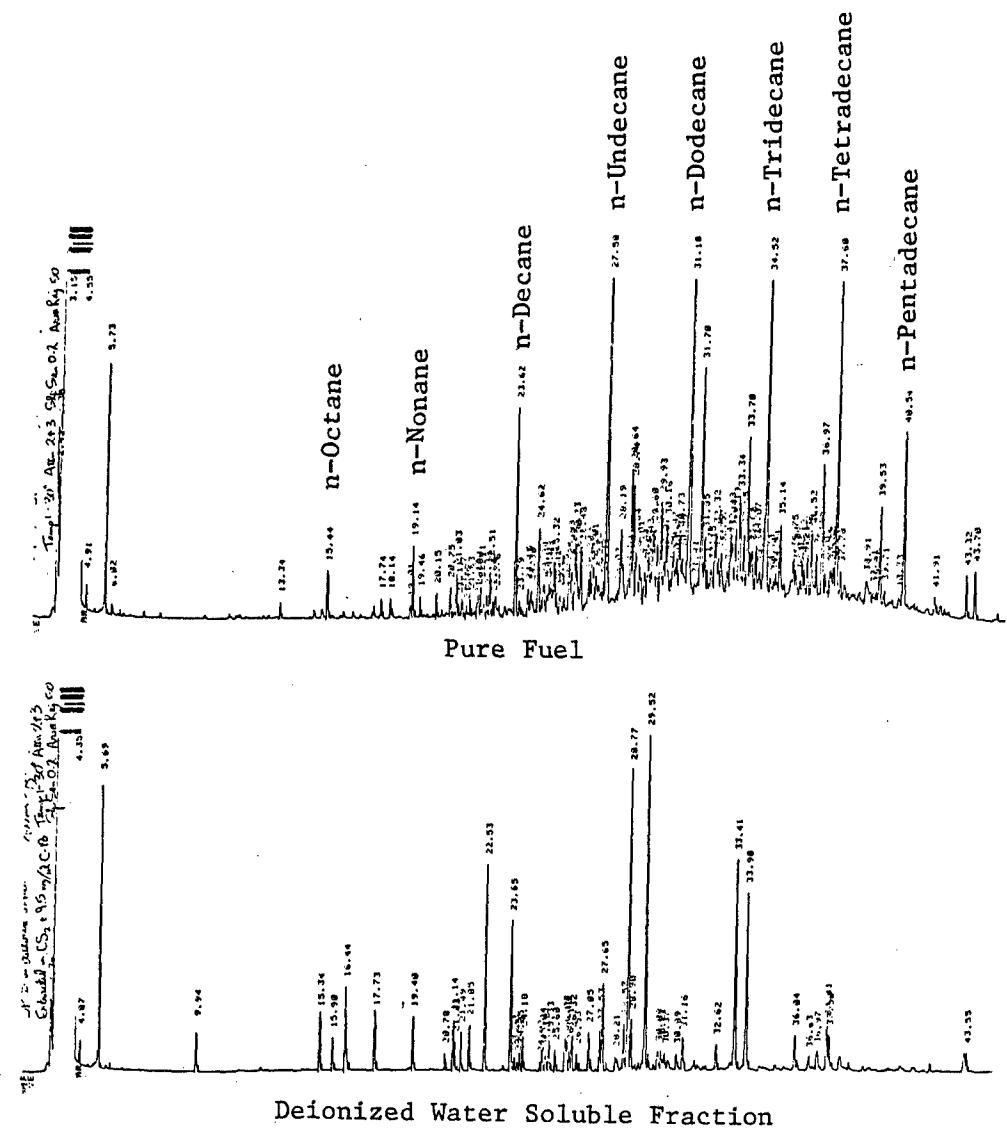
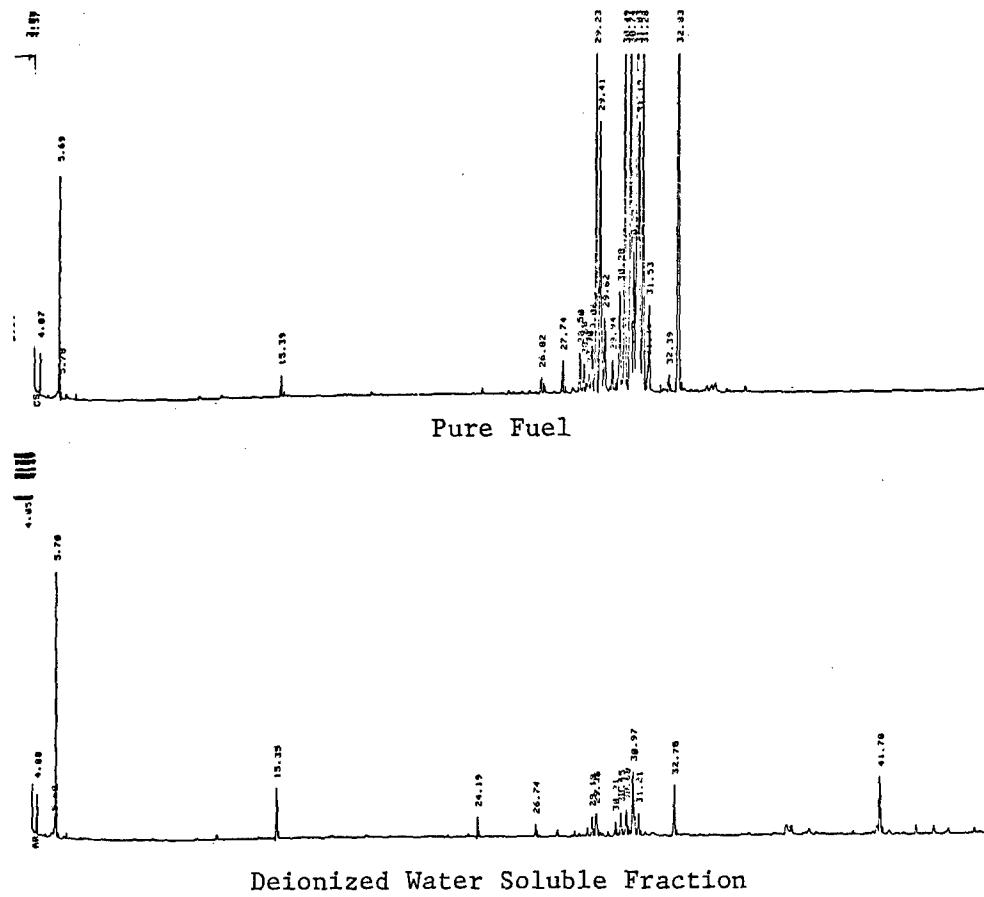


Figure 3. GC traces of JP-8 in CS_2 .



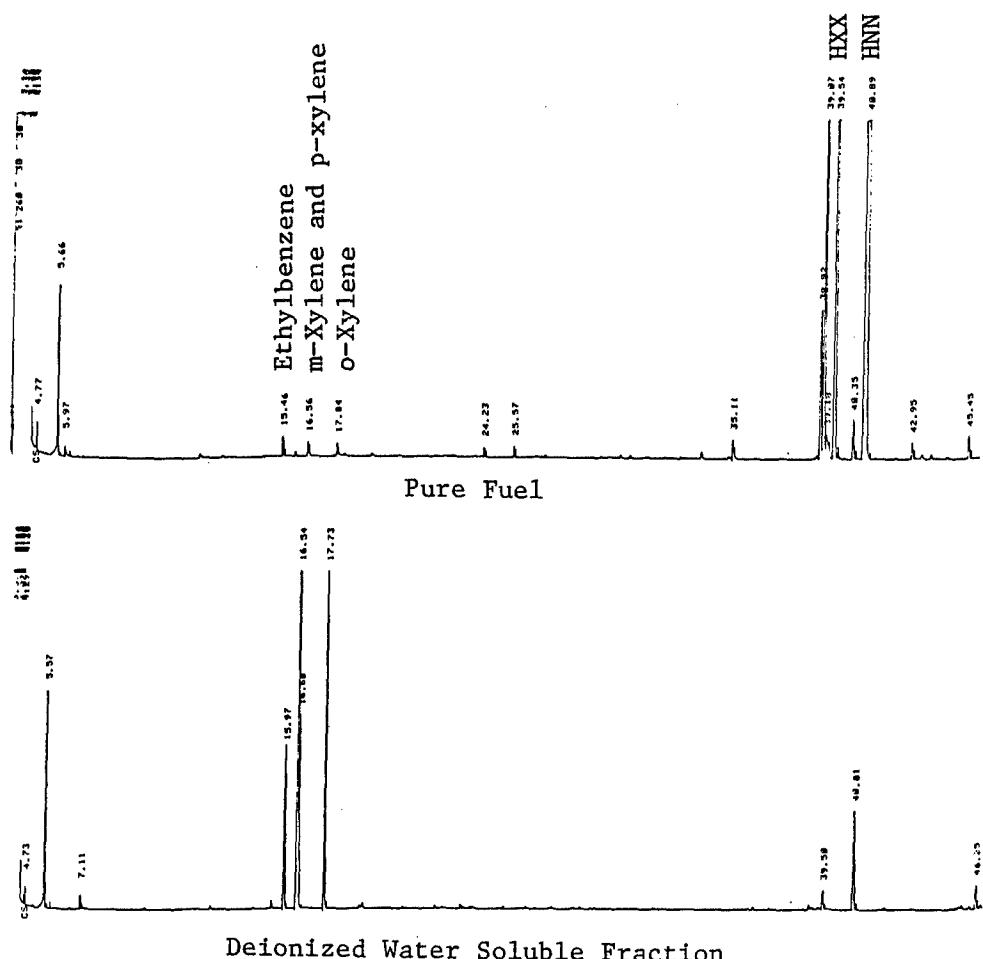


Figure 5. GC traces of RJ-5 in CS_2 .

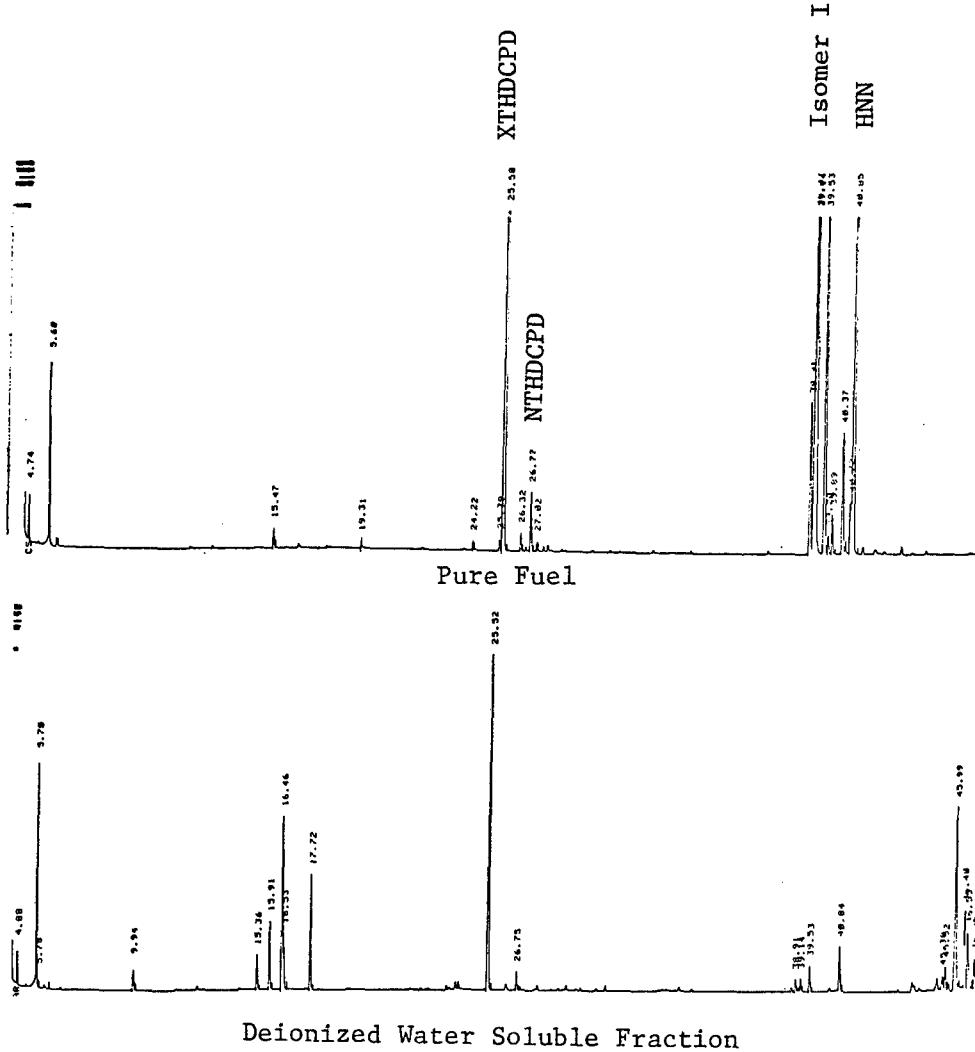


Figure 6. GC traces of RJ-6 in CS_2 .

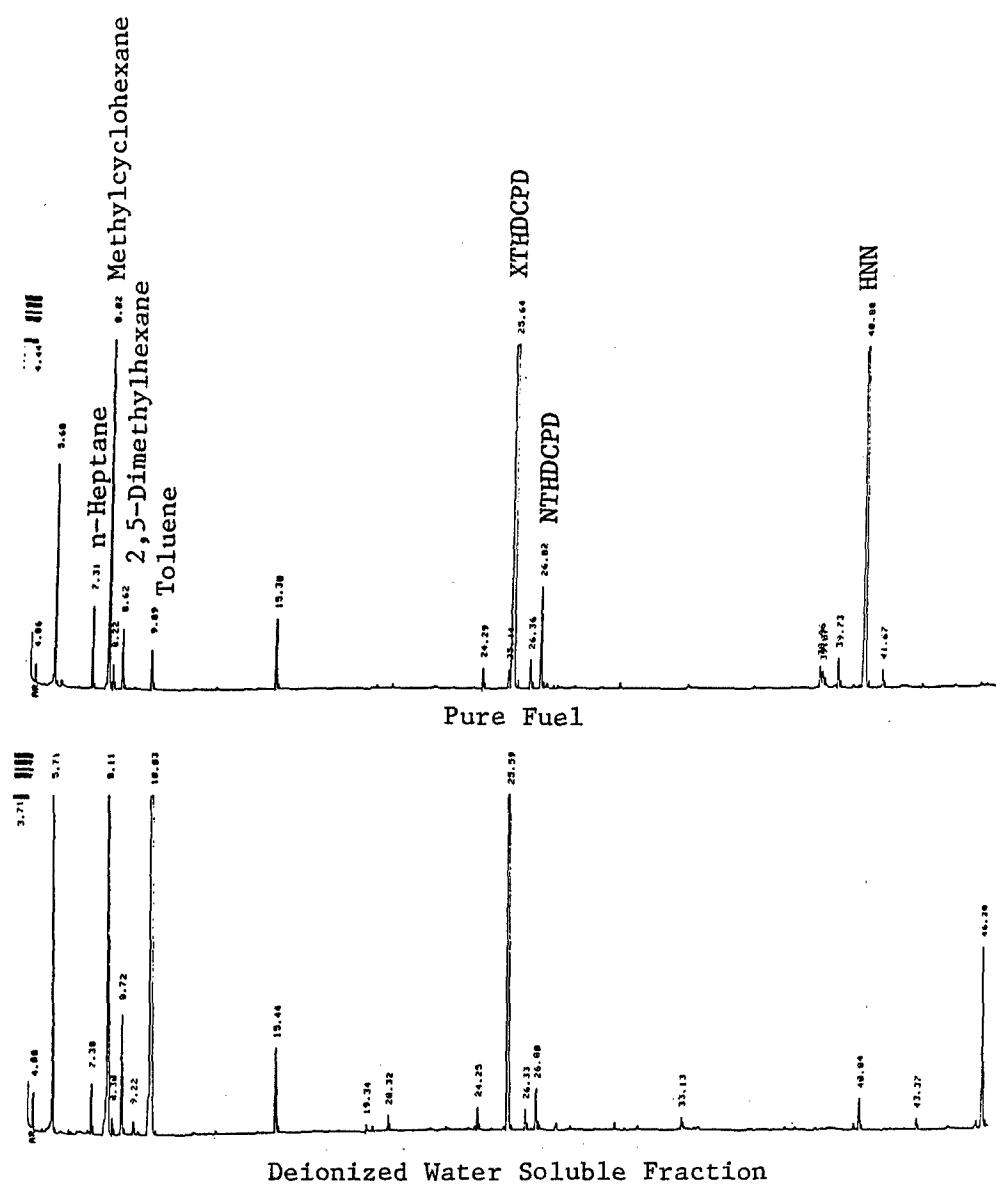
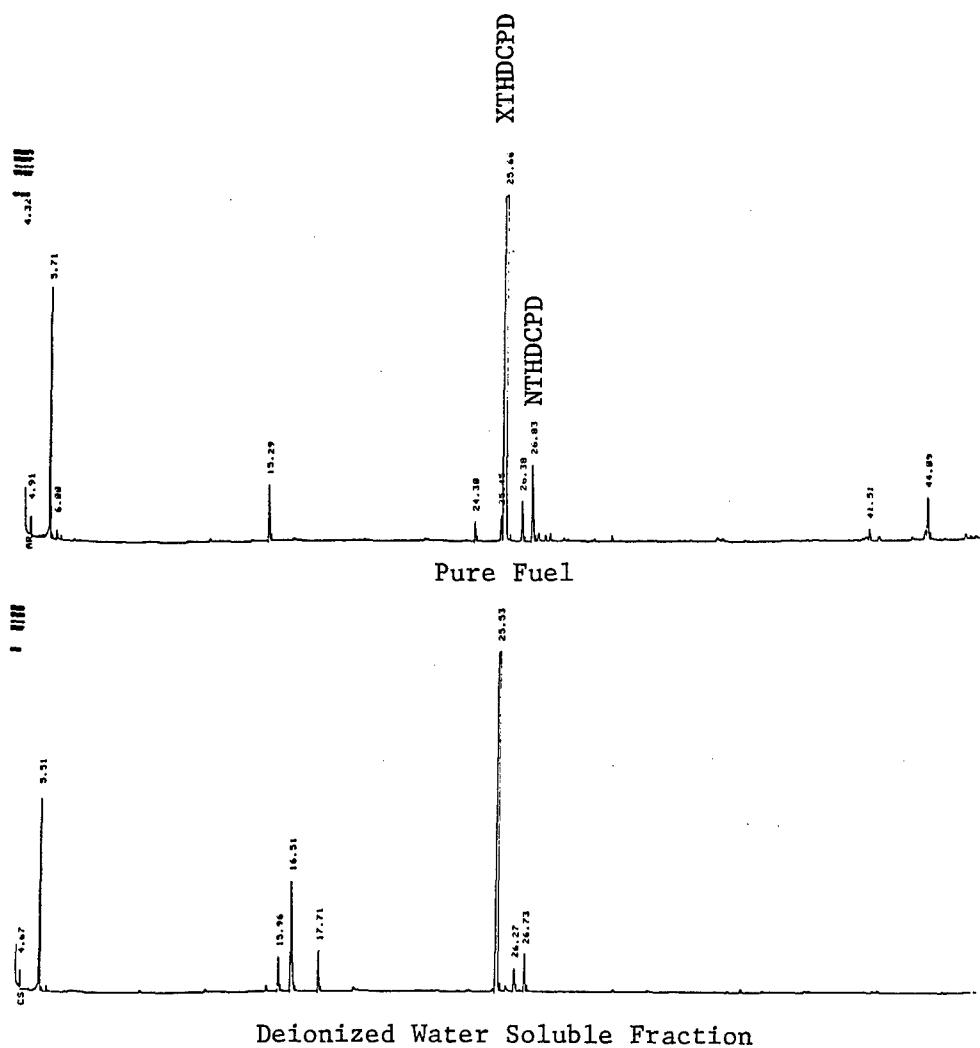
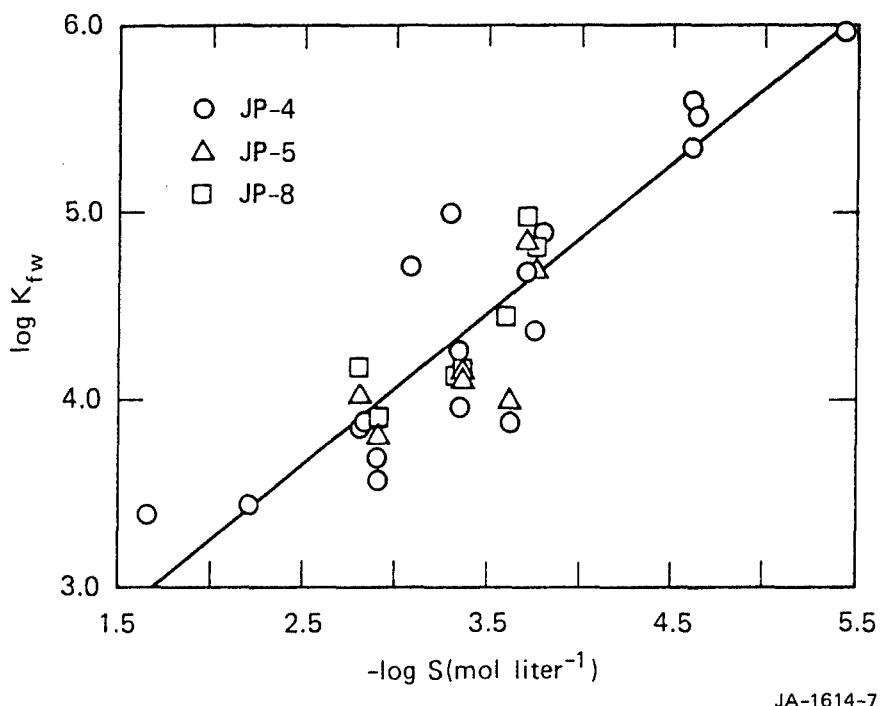


Figure 7. GC traces of JP-9 in CS₂.





JA-1614-7

Figure 9. Aqueous solubility of the pure distillate fuel component (S) as a function of the fuel-water partition coefficient (K_w). (Fuel:water = 1:1000, 20°C)

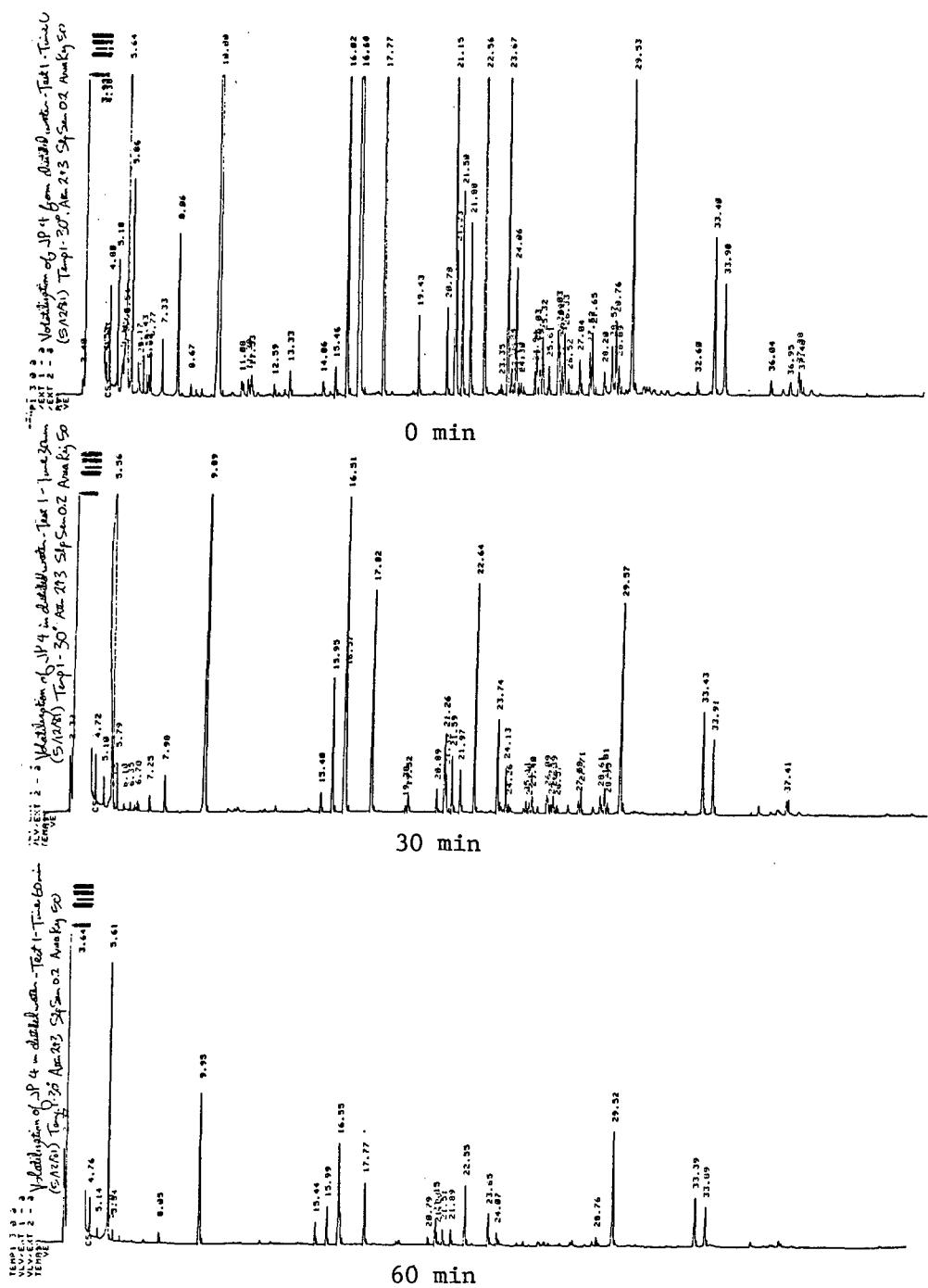
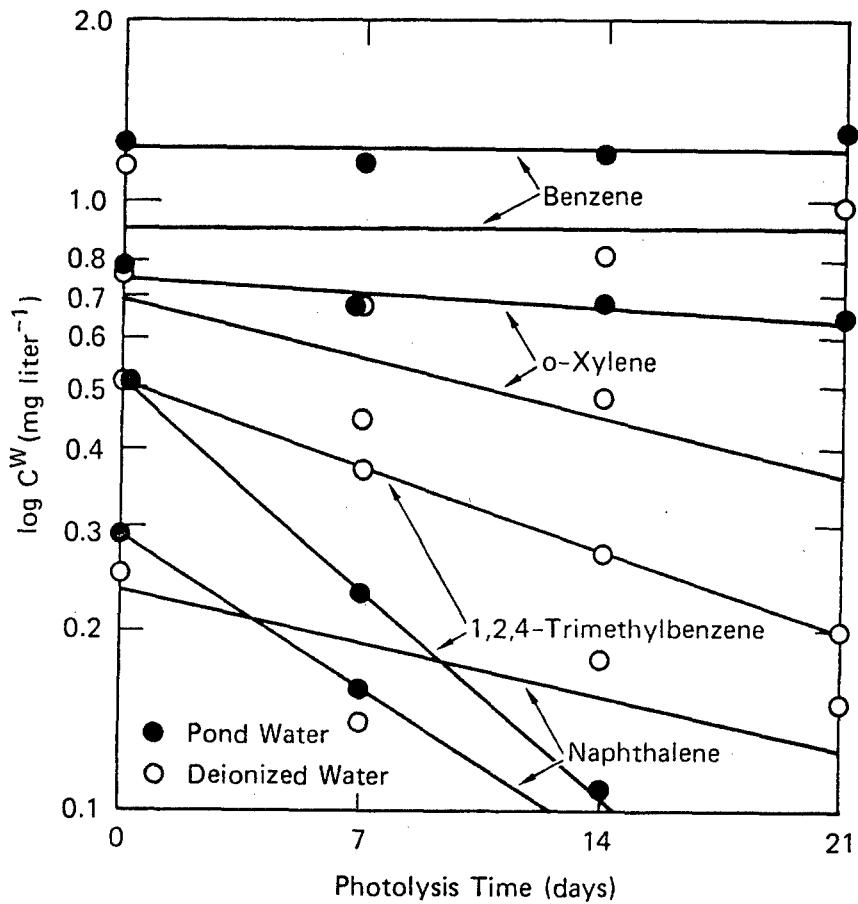


Figure 10. Volatilization of JP-4 fuel components from deionized water (1:1000, 20°C).



JA-1614-6

Figure 11. Photolysis of selected aromatic water-soluble fuel components of JP-4.

TABLE 1. CONCENTRATIONS OF WATER-SOLUBLE COMPONENTS OF JP-4 (mg liter⁻¹)

	Fuel-To-Water Ratio											
	1:10		1:100		1:1000				1:10,000			
	Deion. ^a (20°C)	Sea. ^b (20°C)	Deion. (20°C)	Sea. (20°C)	Deion. (4°C)	Sea. (4°C)	Deion. (20°C)	Sea. (20°C)	Deion. (30°C)	Sea. (30°C)	Deion. (20°C)	Sea. (20°C)
Benzene	9.82	9.06	6.99	4.86	1.41	1.50	1.55	1.32	1.33	0.94	0.07	0.02
Cyclohexane	0.65	0.54	0.48	0.32	0.46	0.39	0.19	0.25	0.31	0.14	0.22	0.07
Toluene	8.49	7.09	7.79	4.95	3.78	3.50	3.71	3.00	3.23	2.64	0.70	0.50
Ethylbenzene	0.67	0.53	0.64	0.40	0.54	0.46	0.59	0.38	0.44	0.42	0.17	0.12
m-Xylene	2.01	1.41	1.83	1.10	{ 1.97 }	1.30	1.89	1.06	1.28	1.22	0.55	0.35
p-Xylene	0.41	0.46	0.49	0.33		0.37	0.36	0.31	0.35	0.34	0.10	0.11
o-Xylene	1.21	0.95	1.17	0.74	0.91	0.79	1.08	0.67	0.78	0.78	0.27	0.21
1-Methyl-3-ethylbenzene	0.28	0.21	0.26	0.16	0.29	0.23	0.36	0.18	0.23	0.24	0.17	0.12
1,2,4-Trimethylbenzene	0.67	0.49	0.63	0.38	0.68	0.53	0.87	0.44	0.57	0.60	0.40	0.28
n-Decane	0.30	0.21	0.27	0.16	0.29	0.23	0.37	0.19	0.25	0.28	0.15	0.11
Naphthalene	0.39	0.29	0.31	0.22	0.27	0.22	0.41	0.24	0.30	0.32	0.10	0.08
2-Methylnaphthalene	0.16	0.11	0.11	0.05	0.12	0.09	0.19	0.10	0.17	0.15	0.10	0.06
1-Methylnaphthalene	0.08	0.06	0.06	0.05	0.08	0.06	0.13	0.06	0.11	0.10	0.06	0.04

^aDeion. = deionized water.

^bSea. = artificial seawater.

TABLE 2. CONCENTRATIONS OF WATER-SOLUBLE COMPONENTS OF JP-8 (mg liter⁻¹)

Fuel Component	Fuel-to-Water Ratio											
	1:10		1:100		1:1000				1:10,000			
	Deion. ^a (20°C)	Sea. ^b (20°C)	Deion. (20°C)	Sea. (20°C)	Deion. (4°C)	Sea. (4°C)	Deion. (20°C)	Sea. (20°C)	Deion. (30°C)	Sea. (30°C)	Deion. (20°C)	Sea. (20°C)
Toluene	0.15	0.14	0.13	0.08	0.05	0.04	0.03	0.03	0.05	0.06	0.02	0.01
Ethylbenzene	0.05	0.04	0.04	0.03	0.03	0.02	0.02	0.01	0.03	0.02	0.01	<0.01
m-Xylene and p-xylene	0.15	0.12	0.13	0.09	0.09	0.07	0.07	0.04	0.08	0.06	0.03	0.02
o-Xylene	0.11	0.09	0.10	0.07	0.06	0.05	0.05	0.03	0.06	0.05	0.01	0.01
1,2,4-Trimethylbenzene	0.20	0.16	0.18	0.11	0.16	0.11	0.15	0.08	0.15	0.10	0.07	0.06
n-Decane	0.15	0.12	0.13	0.08	0.12	0.09	0.12	0.06	0.11	0.08	0.05	0.04
1,2,3,4-Tetramethylbenzene	0.29	0.21	0.24	0.13	0.25	0.17	0.25	0.12	0.23	0.15	0.15	0.12
Naphthalene	0.41	0.34	0.31	0.22	0.25	0.19	0.31	0.20	0.28	0.22	0.06	0.06
2-Methylnaphthalene	0.34	0.27	0.17	0.10	0.15	0.11	0.19	0.11	0.18	0.13	0.08	0.06
1-Methylnaphthalene	0.15	0.12	0.12	0.08	0.13	0.09	0.16	0.10	0.15	0.11	0.07	0.05

^aDeion. = deionized water.

^bSea. = artificial seawater.

TABLE 3. CONCENTRATIONS OF WATER-SOLUBLE COMPONENTS OF JP-9 (mg liter⁻¹)

Fuel Component ^a	Fuel-To-Water Ratio											
	1:10		1:100		1:1000				1:10,000			
	Deion. ^b (20°C)	Sea. ^c (20°C)	Deion. (20°C)	Sea. (20°C)	Deion. (4°C)	Sea. (4°C)	Deion. (20°C)	Sea. (20°C)	Deion. (30°C)	Sea. (30°C)	Deion. (20°C)	Sea. (20°C)
A	0.05	0.02	0.04	0.02	0.05	0.03	0.02	0.01	0.02	0.01	<0.01	<0.01
B	1.47	0.84	1.33	0.63	1.46	1.04	0.81	0.32	0.85	0.59	0.01	0.03
C	0.13	0.07	0.13	0.06	0.13	0.09	0.07	0.03	0.08	0.05	<0.01	<0.01
D	6.81	4.37	6.33	3.36	3.39	2.81	2.38	1.29	2.44	1.98	0.29	0.35
E	1.37	0.80	1.28	0.67	1.46	0.94	1.12	0.53	1.26	0.83	1.27	1.06

^aTentative assignments are Compound A = n-heptane, B=methylcyclohexane, C=2,5-dimethylhexane, D=toluene, E=XTHDCPD isomer of dicyclopentadiene.

^bDeion. = deionized water.

^cSea. = artificial seawater.

TABLE 4. VOLATILIZATION RATE DATA FOR THE MAJOR WATER-SOLUBLE COMPONENTS OF THE DISTILLATE FUELS

Fuel Component	JP-4				JP-8				Average k_v^C/k_v^0
	$k_v^0 = 2.81 \text{ hr}^{-1}$	$k_v^0 = 5.82 \text{ hr}^{-1}$	$k_v^0 = 15.1 \text{ hr}^{-1}$	Average k_v^C/k_v^0	$k_v^0 = 2.71 \text{ hr}^{-1}$	$k_v^0 = 4.33 \text{ hr}^{-1}$	$k_v^0 = 17.0 \text{ hr}^{-1}$	Average k_v^C/k_v^0	
Benzene	0.52	0.32	0.30	0.38 ± 0.12	--	--	--	--	
Cyclohexane	0.65	0.50	0.55	0.57 ± 0.08	--	--	--	--	
Toluene	0.64	0.50	0.80	0.65 ± 0.15	0.37	0.74	--	0.56 ± 0.26	
Ethylbenzene	0.64	0.51	0.56	0.57 ± 0.07	--	--	--	--	
m-Xylene and p-Xylene	0.61	0.48	0.86	0.65 ± 0.19	0.28	0.76	0.82	0.62 ± 0.30	
o-Xylene	0.58	0.46	0.73	0.59 ± 0.14	0.29	0.75	0.51	0.52 ± 0.23	
1-Methyl-3-ethylbenzene	0.60	0.49	0.54	0.54 ± 0.06	0.35	0.68	--	0.52 ± 0.23	
1,2,4-Trimethylbenzene	0.57	0.45	0.74	0.59 ± 0.15	0.26	0.62	0.48	0.45 ± 0.18	
n-Decane	0.56	0.43	0.46	0.48 ± 0.07	0.26	0.59	0.48	0.44 ± 0.17	
1,2,3,4-Tetramethylbenzene	0.54	0.42	0.46	0.47 ± 0.06	0.24	0.47	0.44	0.38 ± 0.13	
Naphthalene	0.30	0.24	0.51	0.35 ± 0.14	0.22	0.28	0.20	0.23 ± 0.04	
2-Methylnaphthalene	0.32	0.26	0.58	0.39 ± 0.17	0.22	0.32	0.25	0.26 ± 0.05	
1-Methylnaphthalene	0.27	0.21	0.25	0.24 ± 0.03	0.21	0.25	0.20	0.22 ± 0.03	

TABLE 5. VOLATILIZATION RATE DATA FOR JP-9

<u>Fuel Component^a</u>	k_v^C/k_v^0			Average k_v^C/k_v^0
	$k_v^0 = 2.87 \text{ hr}^{-1}$	$k_v^0 = 5.91 \text{ hr}^{-1}$	$k_v^0 = 13.7 \text{ hr}^{-1}$	
A	0.41	0.38	0.66	0.48 ± 0.15
B	0.73	0.44	0.51	0.56 ± 0.15
C	0.43	0.39	0.57	0.46 ± 0.09
D	0.38	0.36	0.43	0.39 ± 0.04

^aTentative assignments are Component A = methylcyclohexane, B = 2,5-dimethylhexane, C = toluene, D = XTHDCPD isomer of dicyclopentadiene.

TABLE 6. PHOTOLYSIS OF JP-4 IN WATER

Fuel Component	Fuel Component Concentration (mg liter ⁻¹)											
	Deionized Water				Artificial Seawater				Pond Water			
	Control*	7 Days	14 Days	21 Days	Control	7 Days	14 Days	21 Days	Control	7 Days	14 Days	21 Days
Benzene	1.15	0.68	0.82	0.98	0.76	0.88	0.67	0.65	1.24	1.16	1.20	1.32
Cyclohexane	0.23	0.15	0.17	0.26	0.10	0.11	0.09	0.08	0.31	0.28	0.34	0.34
Toluene	2.82	1.75	1.95	2.01	2.22	2.61	2.07	1.95	2.86	2.74	2.80	2.85
Ethylbenzene	0.40	0.25	0.27	0.25	0.36	0.42	0.33	0.30	0.40	0.40	0.41	0.40
m-Xylene and p-xylene	1.43	0.87	0.85	0.63	1.30	1.54	1.15	0.99	1.46	1.38	1.34	1.20
o-Xylene	0.74	0.45	0.48	0.37	0.70	0.79	0.63	0.58	0.75	0.68	0.68	0.65
1-Methyl-3-ethylbenzene and 1-Methyl-4-ethylbenzene	0.29	0.16	0.14	0.08	0.27	0.32	0.22	0.18	0.28	0.27	0.27	0.22
1,2,4-Trimethylbenzene	0.51	0.23	0.11	0.01	0.49	0.31	0.05	--	0.52	0.37	0.27	0.10
n-Decane	0.23	0.13	0.09	0.03	0.23	0.23	0.15	0.10	0.24	0.19	0.18	0.14
Naphthalene	0.25	0.14	0.18	0.15	0.26	0.06	<0.01	--	0.29	0.16	0.09	0.01
2-Methylnaphthalene	0.10	0.04	0.05	0.02	0.10	<0.01	<0.01	--	0.11	0.03	0.02	--
1-Methylnaphthalene	0.07	0.03	0.04	0.01	0.07	<0.01	<0.01	--	0.08	0.03	0.02	--

*Average of 7, 14 and 21 day dark control samples.

MICROBIAL DEGRADATION OF XENOBIOTIC COMPOUNDS*

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INTRODUCTION

Microbial degradation of xenobiotic compounds in natural environments is probably the most difficult fate process to study and quantitate. Information necessary to predict biodegradation of a chemical depends on laws of chemistry, the genetic capabilities of the microbial populations, and on conditions in the environment.

We have studied degradation of toxicants under conditions that maintain complexities of the natural environment and associated microorganisms. Studies with (NTA) nitrilotriacetic acid demonstrated that this compound, normally biodegradable in freshwater, persists in estuarine environments. The studies illustrate the complex interactions in natural environments that complicate our understanding of biodegradation mechanisms. Interaction with environmental conditions or lack of genetic capabilities within an environment was demonstrated further when freshwater, but not saltwater, microbial populations were shown to adapt within several days to degrade p-nitrophenol rapidly.

Differences in chemical structure affect degradation of toxic chemicals in natural media with mixed microbial populations. Such structures range from compounds like methyl parathion, which is substantially metabolized, to dimilin, which partially degrades and yields nonbiodegradable products, to Kepone, which persists intact.

* Gulf Breeze Contribution No. 437.

MICROBIAL DEGRADATION OF XENOBIOTIC COMPOUNDS

The runoff that results from application of chemicals to agricultural land (direct application to aquatic bodies for weed and mosquito control or via industrial effluents) poses potential threats to nontarget organisms in aquatic environments. Many of these chemicals, depending on their fate, enter aquatic environments where they could be toxic to estuarine plant and animal species (Butler, 1973). Information on the fate and transport of these chemicals is sparse. The effect of a toxic chemical on an aquatic ecosystem depends on the fate of that chemical. Consequently, testing effects of a toxicant in the laboratory requires a knowledge of the compound's environmental stability.

Interaction between fate and effects testing has become increasingly complex due to decreased use of very persistent polyhalogenated organic chemicals and increased use of a variety of new chemicals that have a finite lifetime in the environment. Laboratory toxicologic studies must now be scrutinized continuously and evaluated in accordance with the information generated on fate and transport.

Environmental processes that affect the fate of an organic chemical in the environment include chemical hydrolysis, photolysis, sorption, and biodegradation¹. Biodegradation is probably the most difficult fate process to study and quantitate since it is carried out by living organisms in a dynamic environment. Bacteria and fungi, due to their catabolic versatility, play major roles in the ultimate degradation of synthetic chemicals that enter the environment. The degradative activity of bacteria and fungi is based on their ability to catalyze the initial steps in degradation to produce metabolites that can enter existing metabolic pathways². In addition, the great species diversity of microorganisms in most environments provides a multitude of biochemical pathways for catalyzing the degradation of many structurally different environmental contaminants.

Cycling of carbon in nature requires the existence of organisms, predominately microorganisms, that can degrade molecules produced by biosynthesis. Microorganisms reproduce very rapidly

¹ Baughman, G. L. and R. R. Lassiter (1978), Prediction of environmental pollutant concentration, In: Estimating the Hazard of Chemical Substances to Aquatic Life, J. Cairns, Jr., K. L. Dickson, and A. W. Maki (eds.), ASTM, Philadelphia, Pennsylvania, pp. 35-54.

² Dagley, S. (1972), Summary, In: Degradation of Synthetic Organic Molecules in the Biosphere, Nat. Acad. Sci. Conf., San Francisco, California, June 12-13.

and have a high rate of mutation that allows them to evolve enzyme systems able to catalyze the metabolism of a great variety of organic structures. In spite of this versatility, there is no valid reason to expect that microorganisms should be able to attack organic compounds that bear little or no structural similarity to naturally occurring organic compounds. Thus, most synthetic chemicals that undergo bio-transformation contain structural features common to naturally occurring organic chemicals³.

Scientific prediction of the fate of a chemical in the environment should be based on an established body of knowledge concerning the chemical's structural similarity (or dissimilarity) to natural compounds and the metabolic capability of microbes in the laboratory and should demonstrate how such capabilities are affected by environmental conditions.

Our understanding of microbial metabolic capabilities in the environment and the fortuitous degradation of synthetic organic chemicals is not adequate to provide accurate predictions about the degradation of most chemical pollutants. Much of our knowledge of the mechanisms employed by microorganisms for the dissimilation of organic compounds comes from studies with organisms isolated from terrestrial and freshwater environments. Little information is available on the mechanisms of degradation of organic chemicals by marine isolates. We do not know if organisms shown to play important roles in degradative processes in terrestrial and freshwater environments will perform similar functions in estuarine and marine environments.

At the U. S. Environmental Protection Agency (EPA) Laboratory in Gulf Breeze, Florida, we have been studying microbial degradative processes in estuarine and marine environments for about 10 years. Several case studies on selected synthetic organic chemicals have produced some interesting results that suggest that degradative processes in estuarine and marine environments may differ significantly from those in terrestrial or other aquatic environments.

Evidence was observed in our laboratories on the fate of nitritoltriacetic acid (NTA), the proposed phosphate substitute in detergents (Figure 1) in estuarine environments (Bourquin and Przybyszewski, 1977). Other studies had shown NTA is biodegradable in river water (Thompson and Duthrie, 1968), sewage treatment plants

3 Gibson, D. T. (1975), Microbial degradation of hydrocarbons, In: Physical and Chemical Sciences Research Report 1: The Nature of Seawater, Dahlem Workshop Report, Ed. D. Goldberg (ed.).

(Swisher et al., 1967), and soils (Tiedje and Mason, 1974). Fate studies conducted with natural water samples from Escambia Bay, Florida, showed that NTA persisted in saline waters (Figure 2). We asked the question: Is this lack of degradation due to a lack of the microorganism's ability to degrade the compound, or is it an inhibition of enzyme activity by some environmental factor(s)? Further tests using natural water of various salinities (5-24^{0/oo}) revealed no loss of NTA due to biodegradation by estuarine bacteria (Bourquin and Przybyszewski, 1977). We observed that NTA-degrading microorganisms were entering the estuarine environment with freshwater. However, NTA degradation did not take place in saline environments. It was not determined whether the NTA-degrading microorganisms failed to survive in saline environments or if the enzymes of surviving microbes were not functional. For certain freshwater microbes, the latter phenomenon has been observed (J. M. Tiedje, personal communication). Interestingly, NTA-degrading bacteria have been observed in ocean environments (35^{0/oo} salinity) (Wm. Gledhill, Monsanto Co., personal communication). Those studies confirmed the lack of NTA-degradation in estuarine environments, but showed this capability in higher (constant) salinity environments. No explanations have been given for this difference within saline environments.

NTA MOLECULE

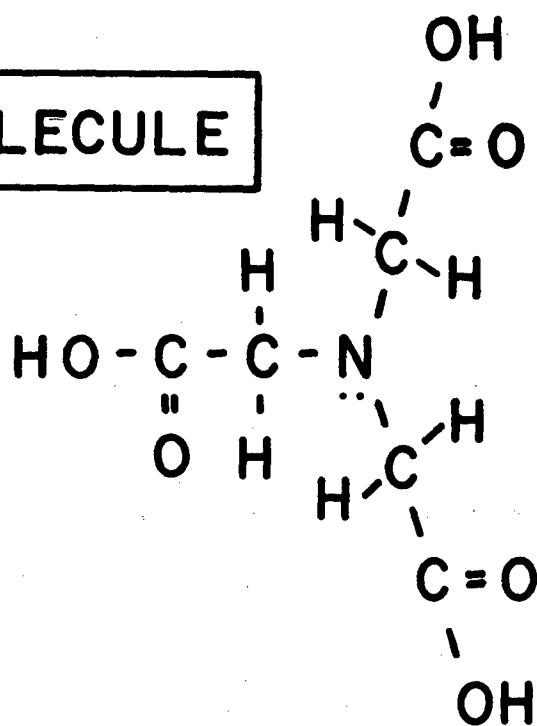


Figure 1. Chemical structure of nitrilotriacetic acid.

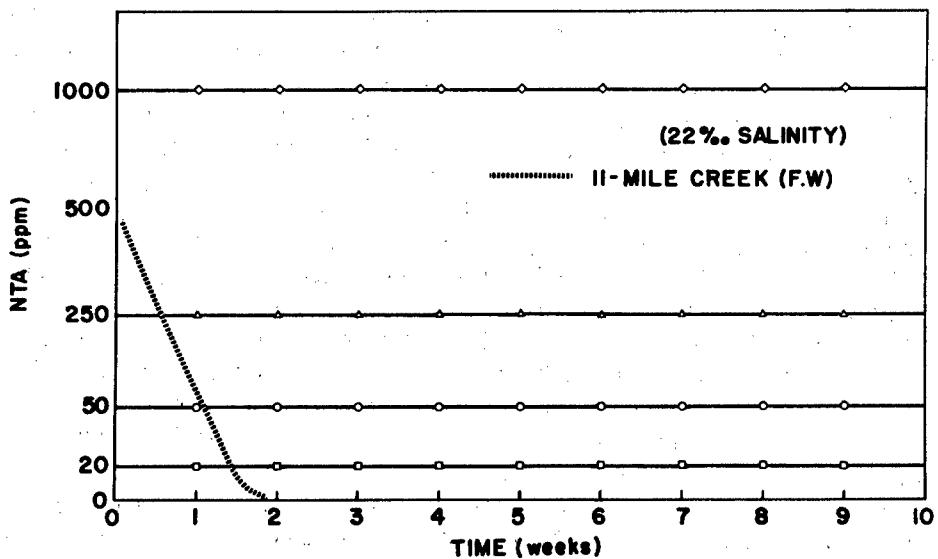


Figure 2. Fate of nitrilotriacetic acid (NTA) at varied concentrations due to biodegradation by indigenous microorganisms in natural water at 22% salinity taken from Escambia Bay, Florida, compared to freshwater samples (11-mile creek).

As our understanding of the microbial processes in aquatic environments has increased, we have developed systems to study them in the laboratory. These systems are designed to maintain some of the complexities of the natural environment (Bourquin et al., 1977). Figure 3 is a schematic of the artificial environment (eco-core) used to study the fate of chemical pollutants. It is a glass cylinder that contains an undisturbed core sample of sediment (50 gm) and the overlying water (200 ml) collected from the field. The cores are brought to the laboratory and maintained at a constant temperature ($25 \pm 1^\circ\text{C}$) and aeration rate (such that the sediment remains undisturbed). For each experiment, the eco-cores are spiked with ^{14}C -labeled toxicant at concentrations below 200 ppb. Four identical sets of cores are prepared and incubated for appropriate intervals. Air exiting each core is monitored for volatile products, using an XAD-4 resin trap for organics and a 1.0 N NaOH trap for $^{14}\text{CO}_2$. To distinguish biologic from nonbiologic processes, identical cores are sterilized with formalin (2% final concentration) and analyzed along with the nonsterile cores. If photolysis is suspected to be a significant factor in decreasing the toxicity of the test compound, light and dark eco-core controls can be included. For comparisons, a set of cores should be prepared with a reference (or benchmark) compound for which fate information is available. We have chosen methyl parathion as our reference.

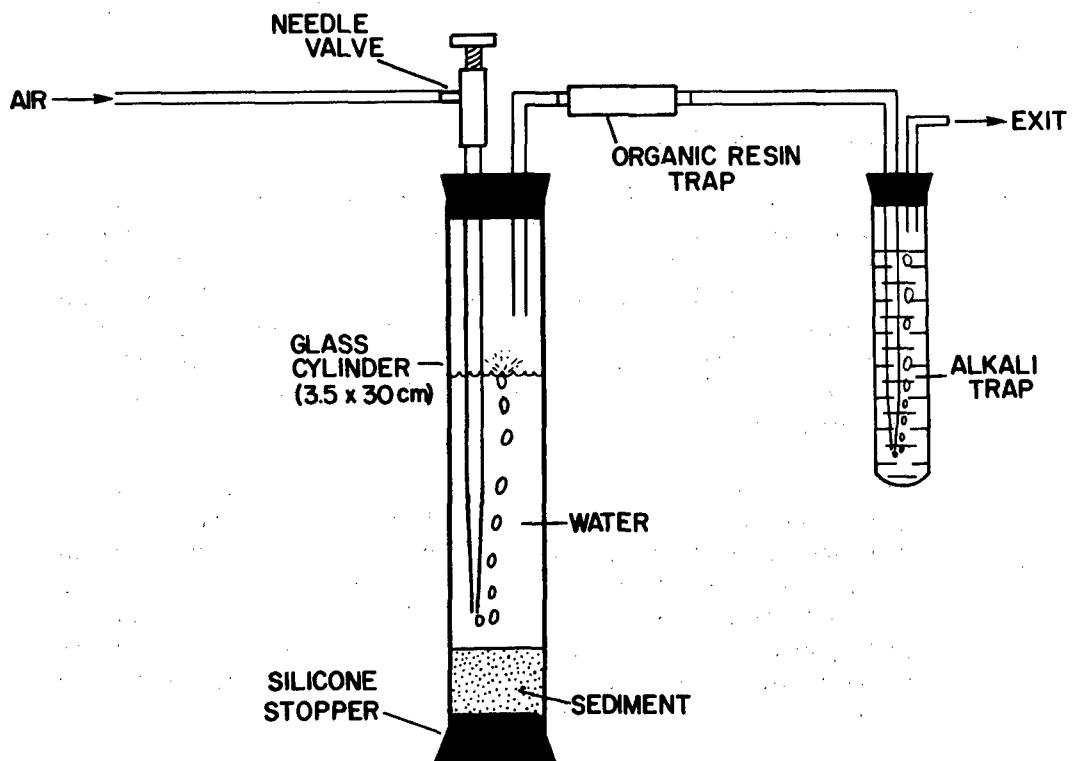


Figure 3. Schematic of "eco-core" fate test system for studying biodegradation.

At appropriate intervals, one core from each experiment set (active, sterile, control, etc.) is dismantled and the distribution of radiolabeled compound and metabolic products in water and sediment is determined by the analytical scheme previously published (Pritchard et al., 1979). From this type of experiment, the following information can be obtained:

- (a) the rate of disappearance of parent compound due to hydrolysis (sterile control), volatilization (XAD resin traps), or adsorption to sediments (chemical extraction), and the extent of parent compound biodegradation (CO_2 production and appearance of polar products);
- (b) the types of degradation products formed in the water column, in interstitial water, and on sediment surfaces; and
- (c) the amount of toxicant residue that becomes tightly bound (solvent unextractable) to sediment.

The use of the eco-core for fate studies has certain advantages:

- (1) It employs natural sediment and water in performing a fate assessment.
- (2) It provides a method for bringing an intact sediment core from the field into the laboratory, thereby assuring that the natural sediment-water interface, where most of the degradative activity occurs, is maintained and the natural complexities of an estuarine sediment/water system are incorporated into the fate assessment.
- (3) It provides fate information based on an integration of all processes that occur in the sediment/water sample. Thus, not only are data supplied on hydrolysis, adsorption/desorption, biodegradation, and volatilization, but also on any process or interaction of processes which contribute to the overall fate and detoxification of the chemical.
- (4) It is a simple system, so easily set up and analyzed that a large number of cores can be employed to permit a variety of environmental parameters to be tested (Bourquin et al., 1979).

Eco-cores have been used as research tools to study microbial interactions with environmental conditions and the degradation potential (genetic capabilities) of microorganisms within an environment. Experiments in our laboratory were devised to determine whether exposure to xenobiotics would cause microbial populations to degrade the compounds faster during subsequent exposures. Studies were performed with water/sediment systems (eco-cores) taken from a salt marsh and a river. Systems were tested for adaptation to the model compounds methyl parathion (MP) and p-nitrophenol (PNP). $^{14}\text{CO}_2$ released from radioactive parent compounds was used as a measure of mineralization. River bacterial populations pre-exposed to PNP at concentrations as low as 45 μM degraded the nitrophenol much faster than did control populations (Figure 4). Likewise with MP, river bacterial populations adapted to a faster degradation; however, higher concentrations of substrate were required. Saltmarsh bacterial populations did not adapt to degrade PNP or MP (Figure 5). p-Nitrophenol-degrading bacteria were isolated from freshwater river samples but could not be detected in saltmarsh samples. These studies show specific biodegradation capabilities in one environment which do not exist in another nearby aquatic environment. The exact

cause of these differences in biodegradation rates is not known; however, there appears to be an absence of microorganisms in the salt water system capable of degrading PNP (Spain et al., 1980).

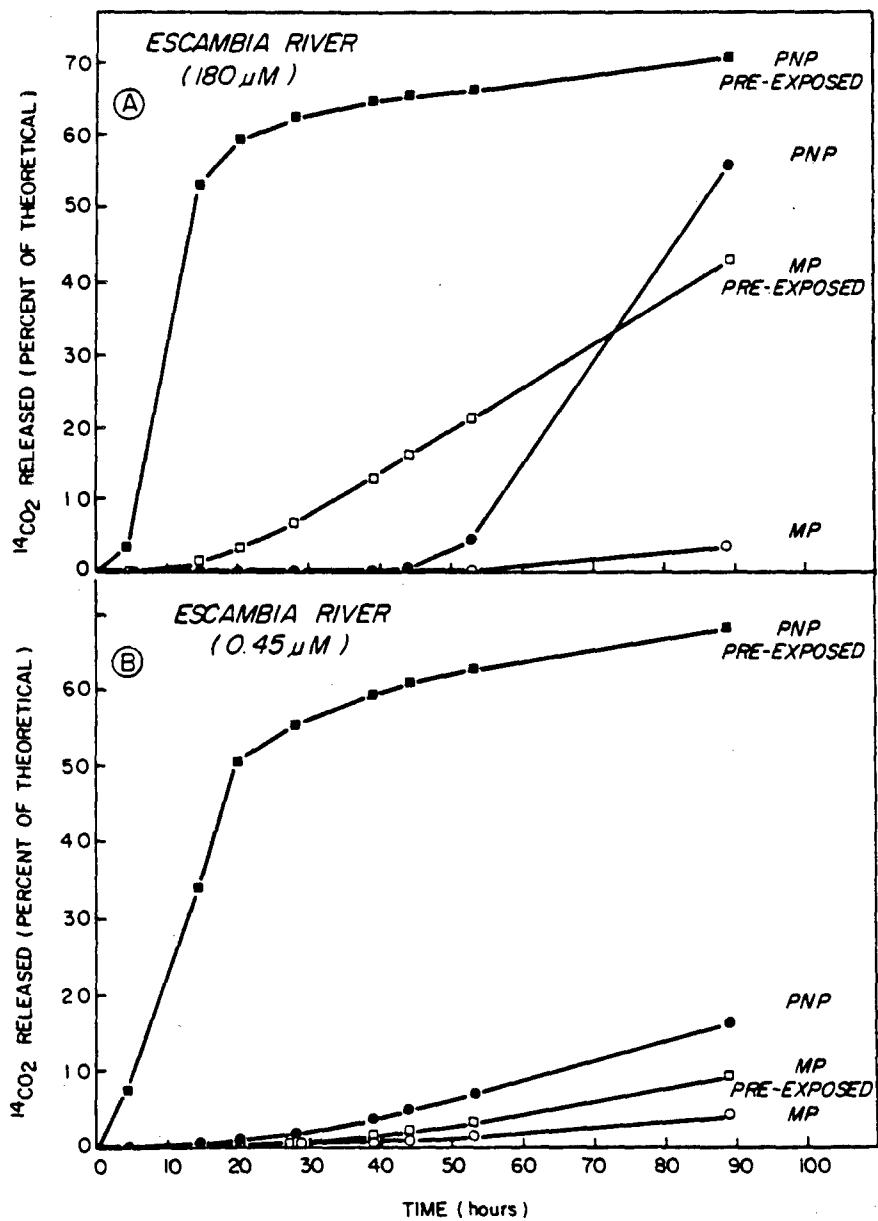


Figure 4. Mineralization in river eco-cores. Cores were supplemented immediately with $[^{14}\text{C}]$ MP or $[^{14}\text{C}]$ PNP, then incubated with aeration at 25°C . $^{14}\text{CO}_2$ released from the cores was trapped and the radioactivity measured. Each compound was tested at concentrations of 0.45 and $180 \mu\text{M}$. (Taken from Appl. and Environ. Microbiol.)

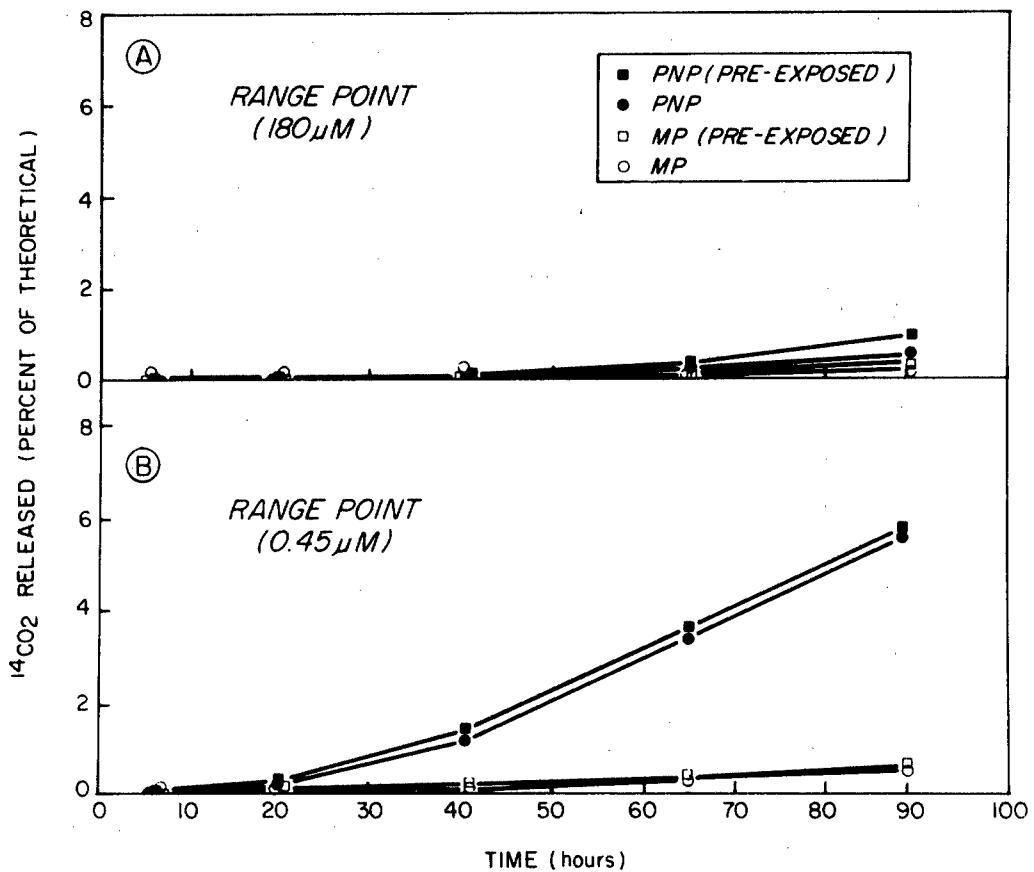


Figure 5. Mineralization in saltmarsh eco-cores. Cores were prepared at the sampling site and spiked immediately with [^{14}C]MP or [^{14}C]PNP at concentration of 180 μM or 0.45 μM . $^{14}\text{CO}_2$ released from the cores was trapped and the radioactivity measured. (Taken from Appl. and Environ. Microbiol.)

As noted earlier, the genetic capabilities of microbial populations have evolved to metabolize most natural organic compounds. Man has recently challenged these capabilities with new, sometimes unique, structural features to which microbes may not have been previously exposed. We have been building a data base on the biodegradation potential of various synthetic organic chemicals in eco-core laboratory fate systems. These compounds are not necessarily selected on the basis of structure, as would be desirable to answer specific questions relating metabolic potential to chemical structure, but more on EPA needs to answer questions on the fate of that particular compound. Nevertheless, the studies produce information which can be used eventually to formulate answers to questions concerning the fate of synthetic pollutants in estuarine environments. Figure 6 shows the structural relationship of some

compounds which have been studied in our laboratory. Methyl para-thion, an organo-phosphate insecticide; carbaryl, a carbamate insecticide; pentachlorophenol, a chlorinated hydrocarbon pesticide, are a few compounds for which we have obtained fate information in eco-core studies.

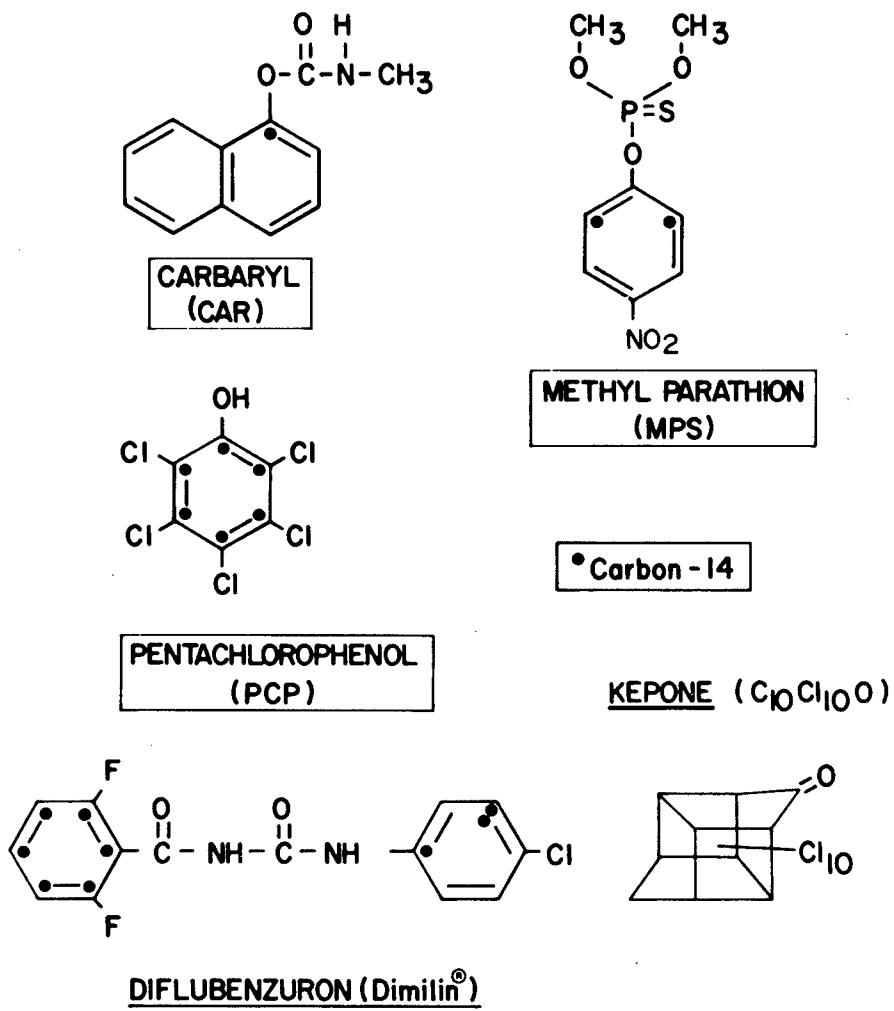


Figure 6. Chemical structure for five pesticides studied in the eco-core systems.

Methyl parathion, our benchmark chemical in all eco-core tests, hydrolyzes to release p-nitrophenol or can be reduced by microbes to aminomethyl parathion (Figure 7). These compounds are eventually degraded to $^{14}\text{CO}_2$ and polar products as the ring is cleaved, p-nitrophenol degrading very readily (Pritchard et al., 1979). These studies suggest microbial reactions that occur in

nature (reduction to aminomethyl parathion), but serve no apparent useful purpose to the metabolizing population. However, the chemical hydrolysis product is readily degraded by the indigenous population and can be used as a source of carbon and energy.

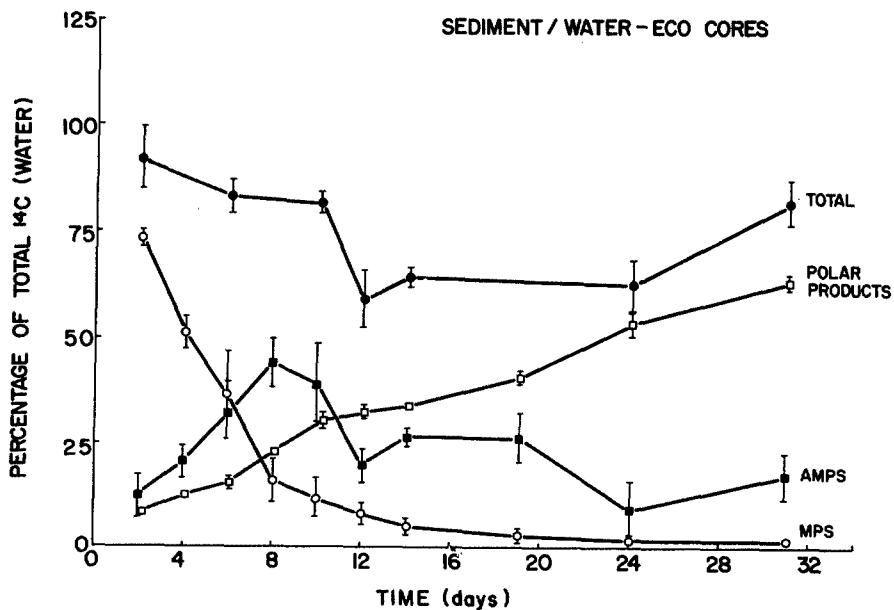


Figure 7. Methyl parathion degradation products identified by thin-layer chromatography in methylene chloride extractable material from water in non-sterile and sterile eco-cores. MPS = methyl parathion, AMPS = aminomethyl parathion.

Figure 8 shows the results of a comparative study of MP, carbaryl, and pentachlorophenol. We have already seen how MP degrades in these systems. Carbaryl is readily converted to α -naphthol which, in turn, is readily metabolized by the indigenous population. Both *p*-nitrophenol and α -naphthol have apparent similarities in structure to natural chemicals. Pentachlorophenol (PCP), on the other hand, is not readily degraded by environmental microbes, although it also contains a hydroxylated benzene structure. Apparently, PCP undergoes a number of fortuitous dechlorination reactions, producing phenolic compounds that can be attacked by the natural microbial populations (A. W. Bourquin and H. L. Fredrickson, 1979, unpublished data). Several dechlorination reactions are known to occur in nature, usually as a result of a rather non-specific enzyme (Zore et al., 1974).

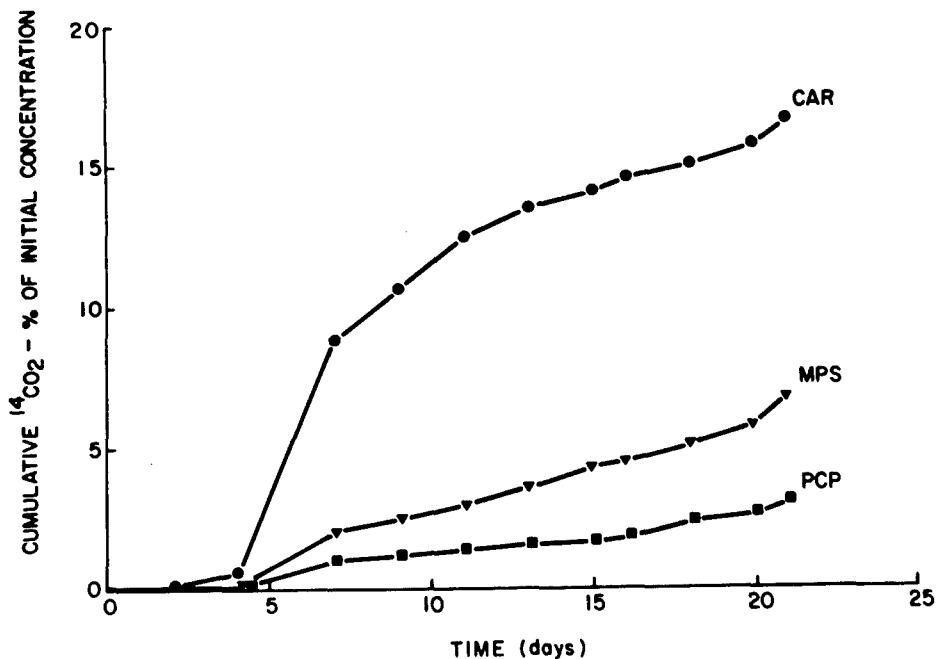


Figure 8. Comparative mineralization rates of carbaryl (CAR), methyl parathion (MPS), and pentachlorophenol (PCP) in saltmarsh eco-cores. $^{14}\text{CO}_2$ was trapped and measured as radioactivity.

In the three examples presented, biodegradation resulted in a partial or complete breakdown of the toxic chemical. Other chemicals, probably due to specific substitutions on the ring(s), are not mineralized. Such a compound is Dimilin. Figure 9 shows the breakdown of Dimilin to difluorobenzoic acid and *p*-nitrophenol urea⁴. There is no further breakdown of these compounds. It previously has been shown that meta-substituted aromatic compounds are less readily degraded than the *ortho* or *para* isomers (Alexander, 1967). It is probable that either the breakdown products do not resemble natural products or the degradative enzymes are inhibited by some substitution. Although some progress in moving away from highly chlorinated persistent chemicals has been made, some of these synthetic chemicals are still produced. Kepone, one such chemical, caused a catastrophic effect in the aquatic environment.

⁴ Pritchard, P. H. and A. W. Bourquin (1979), Fate of dimilin in laboratory estuarine ecosystems, Internal Research Status Report, U. S. Environmental Protection Agency, Gulf Breeze, Florida.

All efforts to show biotransformation of Kepone alone or in conjunction with other organic chemicals did not produce any significant transformation⁵. Moreover, studies in our laboratory have shown that Kepone is toxic to microorganisms (Bourquin et al., 1978) and inhibitory to other biodegradation processes that readily occur in nature. Figure 10 shows the effect of Kepone on MP mineralization in sediment/water cores from the James River, Virginia, and from Northwest Florida. Sediments from the James River estuary were known to be contaminated with Kepone. Results indicate inhibition proportional to Kepone concentration in the sediments. This was confirmed when uncontaminated sediments from a saltmarsh in Northwest Florida were treated with Kepone. These results indicate serious impairment of a degradation process by another pollutant, and emphasize the effects of environmental conditions and other contaminants on the degradation potential of indigenous microorganisms.

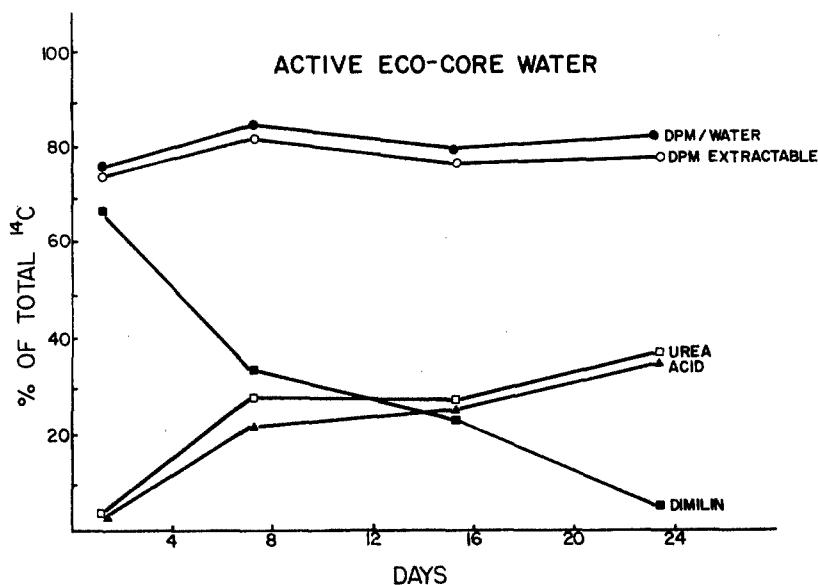


Figure 9. Fate of Dimilin in saltmarsh eco-cores incubated at 25°C with aeration. Cores were supplemented with ¹⁴C-Dimilin and water monitored for total radioactivity as well as products (p-chlorophenyl urea and 2,4-difluorobenzoic acid) identified by thin-layer chromatography and measured by radioactivity.

⁵ Garnas, R. L., A. W. Bourquin, and P. H. Pritchard (1977), The fate and degradation of ¹⁴C-Kepone in estuarine microcosms, Internal Report: U. S. Environmental Protection Agency, Gulf Breeze, Florida.

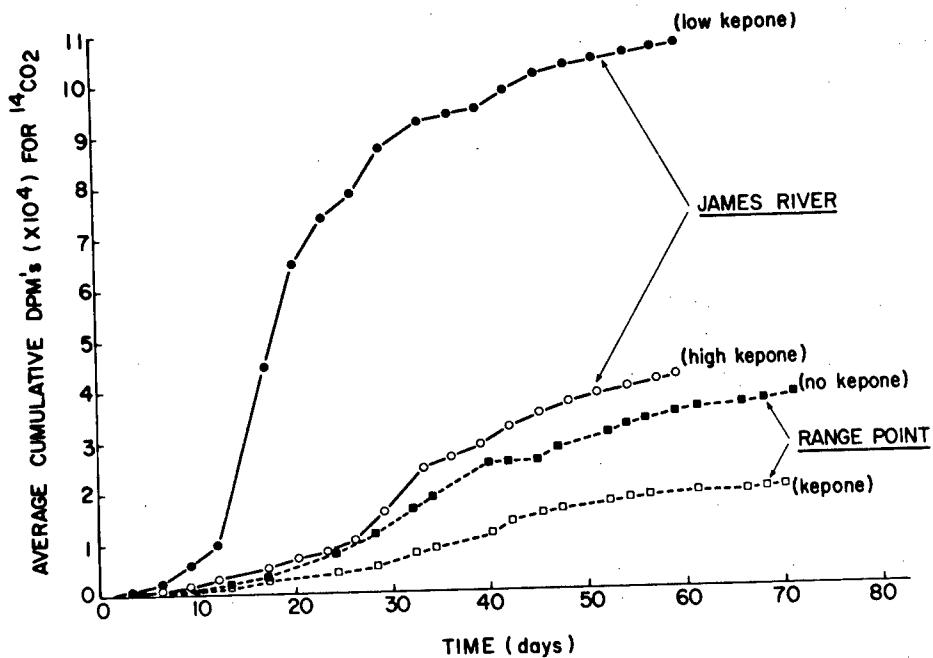


Figure 10. Effects of Kepone on Methyl Parathion (MP) mineralization in saltwater eco-cores. Range Point (Florida saltmarsh) eco-core sediments were supplemented immediately with 500 ppm Kepone and the water treated with 200 ppb ^{14}C -MP. James River cores were constructed with Kepone-contaminated sediments (high = 600 ppm Kepone, low = 140 ppm Kepone) from sites in the James River Estuary and Range Point water which was treated with 200 ppb ^{14}C -MP. $^{14}\text{CO}_2$ was trapped and measured as radioactivity.

It is probably apparent from this brief summary that we must answer many questions before we can accurately predict the action or reaction of natural microbial populations to synthetic chemical pollutants. We have made significant progress in identifying and attempting to answer some of those questions, but others remain elusive. With continued effort, we hope to develop a data base on the fate of synthetic chemicals in natural environments coupled with a better understanding of how and why microbial populations function as they do in nature. With this basic understanding we will be able to predict the fate of toxic chemicals in any environment. I will close by quoting statements of Dr. Stanley Dagley at a workshop on microbial degradation held in Gulf Breeze: "When

a system is complicated, only a genius is able to ask simple questions that are not naive, or develop simple procedures of investigation that avoid artifacts. One complication arises from the fact that the questions themselves alter with time" (Dagley, 1979).

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**UTILIZATION OF AQUATIC ORGANISMS FOR CONTINUOUSLY
MONITORING THE TOXICITY OF INDUSTRIAL WASTE EFFLUENTS**

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Significant improvements in water quality may occur as the discharges of waste effluents into receiving bodies of water comply with current standards limiting the release of both conventional and toxic pollutants. Yet even the best-run industrial plant or waste treatment facility may still be subject to the mechanical or human failures that can cause toxic waste spills dangerous to aquatic communities and public health. Assuming that some toxic spills will occur in spite of preventive measures, the capability for immediate detection of the problem would allow corrective action to be taken and perhaps result in shunting the toxic materials into a holding basin. Although an array of on-line physical-chemical sensors are commercially available for continuous monitoring of wastewater quality, they may not, by themselves, be adequate for determining the actual toxicity of a waste. The premise of this paper is that aquatic organisms themselves may be used to continuously monitor waste toxicity. The requirements for such a work approach are discussed, data from a field test of one system are described, and subsequent improvements in one type of monitoring system are detailed. It should be stressed that toxicity monitoring systems using aquatic organisms as discussed here are primarily intended for the protection of aquatic organisms, not public health, and also that these systems are to be used in conjunction with and not to replace physical-chemical monitoring devices.

The inadequacy of nonbiological sensors for detecting toxic waste conditions is supported by several lines of evidence (Cairns and van der Schalie, 1980). In some cases, toxic materials may cause adverse biological responses at concentrations below detection limits attainable with present monitoring equipment. For example, a recommended water quality criterion for the protection of aquatic

organisms from elemental phosphorous was set at 0.01 $\mu\text{g/L}$ (Sullivan et al., 1979). By contrast, the only readily available analytical technique (gas chromatography) had a maximum detection limit of 0.05 $\mu\text{g/L}$. Neutron activation can go below the 0.01 $\mu\text{g/L}$ concentration limit, but only if interfacing substances are not present in the water. A second drawback of physical-chemical sensors relates to the complexities associated with most waste effluent discharges. It is more likely that there will be many toxicants in a waste effluent instead of just one, and the biological interactions resulting from the mixture (including possible synergism or antagonism) are not likely to be predictable from analytical measurements, even if all toxic components are included. Temporal variations in the composition of the waste could also influence toxicity in unpredictable ways, as could the addition of unknown materials from sources upstream of the waste discharge point. Finally, many water quality variables such as temperature, pH, hardness, etc., can have very large effects on the toxicity of material to aquatic organisms. One well-known example is the reduction in toxicity of many heavy metals (zinc, copper, and others) with increasing water hardness. Knowledge of the concentration of a toxicant alone may thus be inadequate for a proper assessment of its impact on aquatic organisms. Many of these deficiencies could be overcome if biological data were collected along with continuously monitored physical-chemical data.

Although there are many biological monitoring schemes that could be used to evaluate waste effluent toxicity, the primary emphasis here will be on "in-plant" units used at an industrial or other site to continuously monitor a discharged material. (Coupling of "in-plant" with "in-stream" monitors is discussed in Cairns, 1975a,b and Cairns et al., 1972, 1973a,b.) Basic characteristics of "in-plant" monitors discussed in this paper include the following: (1) aquatic organisms used as monitors are held under controlled conditions and are exposed on a frequent or continuous basis to the wastewater being tested; (2) a specific physiological or behavioral parameter of the organisms is monitored by a device capable of producing an appropriate response should the organisms exhibit abnormalities; (3) the organisms are used for detection of developing acutely toxic conditions in the waste--rapid detection of low concentrations of toxic materials having chronic or cumulative effects is not likely.

Different types of toxicity monitoring devices fitting the above criteria have been developed and are reviewed extensively in Kingsbury and Rees (1978), Cairns and van der Schalie (1980), and Cairns and Gruber (1980). Many share the same basic components, including a wastewater delivery system to provide either pure or diluted effluent to the test organisms, mechanical or electronic transducers capable of measuring the biological parameter(s) being

observed, and a data analysis system, including some mechanism to provide an alarm in case of developing toxic conditions. In order for any monitoring system to function effectively, five basic conditions must be met. A summary of these conditions is given below (Cairns and van der Schalie, 1980).

1. Selection and Measurement of Useful Biological Responses

The earliest types of continuous toxicity monitoring systems relied on visual indications of stress or mortality in fish placed in flowing water or wastewater (Henderson and Pickering, 1963; Jackson and Brungs, 1966). Obvious problems with this approach include the need for continual visual observation and the considerable delay which may occur between exposure of fish to toxic materials and their death. For these reasons, current systems rely on automated devices capable of measuring sublethal symptoms of poisoning. The physiological or behavioral manifestations of such poisoning must therefore be capable of being interfaced to a computer or other electronic recording equipment, but without imposing undue stress on the organism. With fish, such stress could be caused by attachment of electrodes or by undue restraint.

2. Rapid, Reliable Response to Toxic Waste Conditions

The speed of response of a biological monitoring system to developing toxic conditions is critical to its successful operation. The primary factor influencing an organism's ability to respond will, of course, be the concentration of the toxicant(s) to which it is exposed. The lower the concentration with respect to acutely toxic levels, the longer the time required for a response. One example of this relationship is shown in Table 1. If these trout were used in a monitoring system, their usefulness with respect to detecting a cyanide spill would depend on the cyanide concentration in the waste, the dilution of the waste (if any) to which the trout were exposed, and the delay time between introduction of the waste into the trout test system and the wastewater discharge into a receiving body of water. If the wastewaters were monitored at the influent to a holding pond rather than just prior to discharge, longer delay times in toxicity detection could be tolerated. Toxicants which cause long-term cumulative effects at low concentrations will most likely not be detected (Brown, 1976), but the purpose of these systems is to rapidly detect high waste toxicity likely to cause acute effects. The form of the toxicant may be as important as its total concentration. Materials sorbed to particulate matter may be unavailable for uptake by organisms and thus not be detected. In addition, materials which must be metabolized by the organism into an active form may slow response times considerably (Koeman et al., 1978).

TABLE 1. THE RELATIONSHIP BETWEEN CYANIDE CONCENTRATION AND TIME TO LOSS OF RHEOTAXIS IN RAINBOW TROUT*

<u>Cyanide (mg/L as CN)</u>	<u>Time to Loss of Rheotaxis (min)</u>
2.0	2.8
0.3	8.8
0.18	24.0
0.14	90.2
0.09	1620.0
0.07	4440.0

* Data from sources cited in Reimann, 1974.

Another potential problem for organisms continuously exposed to wastewaters is loss of sensitivity following long-term exposure to low toxicant concentrations. Cairns et al. (1973b) found that bluegills (*Lepomis macrochirus*) exposed to zinc at 0.01 of the 96-hour LC₅₀ (0.075 mg/L) for 41 weeks exhibited decreased activity responses to a simulated zinc spill (3.0 mg/L), but that changes in ventilatory rate were not affected. In other studies, the oxygen consumption of bluegills (O'Hara, 1971a) and the coughing rate of brook trout, *Salvelinus fontinalis*, (Drummond and Carlson, 1977) both peaked and then decreased toward preexposure levels within 24 hours after the start of their exposures to sublethal concentrations of copper. (A cough is a rapid movement of the ventilatory apparatus in response to an irritation of the gill surface [Hughes, 1975].) Any reduction in the response of toxicity monitoring systems could be minimized through periodic replacement of the test organisms.

3. Minimal Responses to Nonharmful Variations in Water Quality

Certain changes in water quality may elicit responses from organisms when no toxic materials are present. Such variables as temperature, pH, and dissolved oxygen may have to be controlled and/or closely monitored to prevent "false alarms" from occurring. Cairns et al. (1973a,b; 1974) found that bluegills in a pollution monitoring system showed increased breathing and activity rates when the initial diurnal temperature cycle of 24.8 to 26.0°C became 24.8 to 29.2°C. With rainbow trout, sublethal variations in pH between pH 6 and pH 9 caused changes in opercular movements, coughing, and metabolic rates (Hargis, 1976). Changes in dissolved oxygen concentrations could have dramatic effects, especially when fish ventilatory patterns or other respiratory-related parameters are being monitored. These kinds of problems illustrate the fact that biological monitoring systems should be used in conjunction with physical-chemical monitoring systems to discover the reasons for any abnormal responses.

4. Sound Criteria for Determining Toxicant-Related Responses

To determine that an organism is showing abnormal responses requires a statistically sound grasp of the normal range of variability of the parameter being monitored. This means that a preexposure period should be used to define the normal levels (with confidence limits) for each organism (or group of organisms) and each parameter being monitored. An additional set of control organisms which receives no wastewater materials is also appropriate to allow detection of possible changes in dilution water quality. This approach has been used in monitoring systems which utilize fish activity patterns (Cairns et al., 1973a,b; Hall et al., 1975) and ventilatory patterns (Cairns et al., 1973a,b; Morgan and Kuhn, 1974; van der Schalie et al., 1979; Gruber et al., 1978). Parameters having diurnal periodicities may require a different range of normal values for different times of the day.

5. Ease of Operation and Data Interpretation

An electronically-based system could easily provide an alarm mechanism to indicate developing toxic waste conditions. Interpretation of the meaning of the alarm would require follow-up with checks on the monitoring system and on the effluent. Ensuring reliable monitoring system operation may require environmental control (temperature, humidity, etc.), especially if electronics are involved and the monitor is located in an unprotected area. Use of readily-available standard toxicity testing species such as bluegills or rainbow trout may also be desirable, since their response to a large number of toxicants is relatively well-defined and their maintenance prior to testing is not difficult. Advantages of utilizing organisms found in the body of water receiving the wastewater discharge must be weighed against possible difficulties in obtaining and maintaining the animals.

Of the many potential toxicity monitoring systems using aquatic organisms, few have been tested in the laboratory and even fewer have received any actual field testing. So far, most of the systems that have been developed have utilized fish, but bacteria and aquatic invertebrates have also been used. With bacteria, monitored parameters have included biomass (Jeffers and Taylor, 1977), nitrification capability (Holland and Green, 1975; Stroud and Jones, 1975), and respiration (Axt, 1973a,b as described in Morgan, 1976; Reeves, 1976*; Solyom, 1977; Clarke et al., 1977). Of these,

* Reeves, J. B. (1976), Activated sludge system influent toxicity monitoring through utilization of a commercial respirometer, M.S. Dissertation, Virginia Polytechnic Institute and State University, Blacksburg.

oxygen consumption has been most widely used. Oxygen electrodes are generally used to measure the difference in oxygen content between test chamber influent and effluent liquids; under toxic conditions the difference will decrease as microbial metabolism of nutrient materials in the water is inhibited. Solyom (1977) reports that a bacterial respiration monitor has been used to detect copper, cyanide, low and high pH levels, and has detected toxic changes in the effluents from chemical pharmaceutical companies on several occasions.

With aquatic invertebrates, both activity levels and oxygen consumption appear to be usable parameters in toxicity monitors. Arnold and Keith (1976), developed a continuous flow respirometer for larger invertebrates. Activity measurements have been made of the respiratory movements of aquatic insects using strain gauges (Kapoor, 1971), of water currents caused by body movements (Heusner and Enright, 1966), and of electrical signals generated by movements of crayfish in water (Maciorowski, 1977*). The latter arrangement has been used to automatically analyze the responses of crayfish to cadmium. Wire electrodes placed in chambers with eight individual crayfish (Cambarus acuminatus) picked up electrical signals generated by crayfish movement. The signals were amplified and passed to a microcomputer which recorded the number of electrical peaks per unit time, which was proportional to the amount of movement. Data were summed and totals printed out for each crayfish every 15 minutes. Crayfish were monitored for several days prior to toxicant exposure to determine the normal variability in movement patterns for each crayfish. In tests with cadmium, four animals were exposed to the toxicant and four were unexposed controls. Definite responses were registered within 2 hours of exposure at concentrations of 2.5 and 0.5 mg/L, but at 0.1 mg/L no responses were noted until 113 hours after exposure had begun.

Much more work has been done with utilizing fish as toxicity monitors than with bacteria or invertebrates. Devices have been developed using fish movement patterns, rheotactic response, avoidance, oxygen consumption, and ventilatory patterns (primarily opercular movement rates and "cough" rates). An activity monitor which recorded the movement rates of bluegills by counting the frequency with which three photocell beams across a test tank were broken was described by Waller and Cairns (1972). Counts from four fish at each treatment level were stored in counters and automatically photographed each hour. Changes in the variances of the light beam interruption counts were used as an indication of abnormal

* Maciorowski, A. F. (1978), The development and assessment of an automated biological monitoring system using crayfish locomotor activity to detect toxic stress, Ph.D. Dissertation, Virginia Polytechnic Institute and State University, Blacksburg.

movement patterns. (Normal patterns for each fish were determined separately during a preexposure period.) The lowest detectable sublethal concentration of zinc during a 96-hour exposure was between 2.94 and 3.64 mg/L. An improved version of this system utilized a minicomputer to record the photocell beam interruptions every half-hour and store them for later analysis (Westlake et al., unpublished*; Cairns et al., 1975). This system detected sublethal levels of TNT and nitroglycerin production effluents from a U.S. Army munitions plant (Westlake et al., 1974a,b). No significant response was found to changes in acidity in the range between pH 5 and pH 10 (Westlake et al., 1974c). Other fish activity monitors using rainbow trout (Salmo gairdneri) have been developed at the Stevenage (England) Laboratory of the Water Research Centre. These have been used to monitor river waters in England, with alarms set to be triggered when fish activity exceeds a preset level (Acker, 1979). Further operational details are not presently available.

Another parameter related to fish activity is rheotaxis, the ability of a fish to maintain its position in a water current. Poels (1975; 1977) designed an automatic system which employs photocells to determine when loss of rheotaxis occurs in a chamber through which test water is flowing. When any one of the three fish in the chamber falls back to the downstream end of the chamber, photocell beams are broken and a mild electric shock is applied to force the fish back into the upstream area. If two of the three fish spend more than 5 minutes of a 25-minute period in the downstream end or pass into it more often than normal in 15 minutes, an alarm switch is activated. This system has been tested for 18 months using Rhine River water. No toxicant alarms have occurred during this time, and there have been no operational difficulties despite the turbid nature of the river water. Experiments with toxic substances, such as lindane, do show that acutely toxic levels can be detected well before death occurs. In a similar arrangement used by Besch et al. (1977), loss of rheotaxis is measured by the interruption of vertical light beams, and surfacing of the fish is measured by horizontal light beams. Also, a kinetic screen has been added at the downstream end of the chamber. This device produces electrical pulses when touched by the fish and provides a measure of the amount of time spent in the downstream area. Besch employed alternate periods of rest (generally 10 min of slow vertical current) and stress (5 min of strong longitudinal current) instead of a continuous downstream flow of test water. This automatic system detected phenol at sublethal concentrations and DDT at acutely toxic levels.

* Westlake, G. F., J. Cairns, Jr., and I. D. Prather (Unpublished), An automated device for long-term monitoring of respiration and locomotor activity in fish.

Two similar systems have been described, but data on their toxicant detection capabilities were not given. A monitoring system based on fish rheotactic responses was used at a water treatment plant on the Oise River near Paris (Vivier, 1972*). When these fish were unable to swim against water flow after electrical stimulation, an audible alarm was generated. Another unit, which was used at a wastewater treatment plant in Sweden to monitor effluent quality, relied on strong light instead of electrical shock to promote the rheotactic response (Hasselrot, 1975).

A final system based on rheotaxis incorporated a conveyer belt system to transport weakened fish out of the test chamber at which time they are detected by a photocell system. Using eight golden orfe, a response by 50 percent of the fish to 0.1 ppm thiadan was noted after 114 minutes of exposure. A similar fish test alarm device indicated possible toxicity in the water of the Main River (Germany) several days prior to a large fish kill (Scharf, 1979).

More complex locomotor responses to toxicants have been analyzed through computer tracking of fish movement patterns with an array of photocells in a test tank (Kleerekoper et al., 1970, 1972; Kleerekoper, 1977). Photocells (Cripe, 1979) or a video camera (Westlake and Lubinski, 1976; Lubinski et al., 1977), have been used to monitor fish behavior in a preference-avoidance chamber, where the fish are given a choice between clean water and water containing toxicant. Avoidance behavior for a given species may vary widely for different materials; the green sunfish (Lepomis cyanellus) avoided only 8 of 40 toxicants tested in one study (Summerfelt and Lewis, 1967). In addition, the complexity of the behavior patterns displayed by many fish may make rapid detection of toxic materials less likely than with some of the other available monitoring end points.

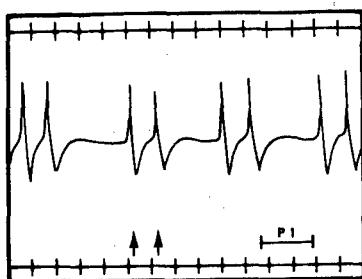
As with bacteria and invertebrates, oxygen consumption of fish has been used to monitor toxicity. In one system, oxygen levels in water going into and coming out of a sealed chamber containing fish was measured; if the difference between the two was too small, death was assumed to have occurred and an alarm was sounded (Kitsutaka, 1974). Electrolytic respirometers used by Callahan (1974) detected significant changes in oxygen consumption in fish exposed to sublethal levels of ammonia and two aircraft firefighting foams. A water flow-through chamber containing individual fish had to be stopped while oxygen levels were measured. Data from each fish were stored on magnetic or paper tape for later analysis.

* Vivier, A. (1972), Monitoring water pollution. The observation post at Boransur-Oise, Sixth Int. Wat. Poll. & Res. Conf., Paper No. 21.

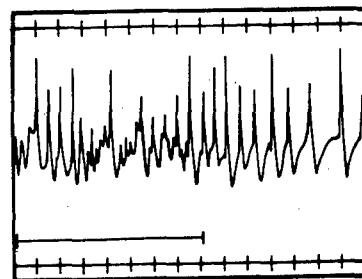
Information relating to oxygen consumption can also be obtained by monitoring the ventilatory patterns of fish. Heath (1972) reviewed the methods available for monitoring fish ventilatory patterns and found that the simplest method and the one causing the least strain on the fish was simply to measure the electrical signals picked up on dual electrodes placed in a tank with the fish. A typical signal generated by bluegills (Lepomis macrochirus) is shown in Figure 1. Many fish, both freshwater and marine, produced similar signals (Cairns et al., 1980). Information on ventilatory rate and depth, cough rate, and whole body movements can be obtained. Most systems capable of automatic monitoring of fish ventilatory signals have concentrated on recording only ventilatory rate. Although coughing appears to be a sensitive measure of the presence of toxic materials in water (Drummond and Carlson, 1977), it is also quite difficult to detect electronically (van der Schalie, 1980). An automated electronic device has been developed which generates a voltage proportional to the ventilatory rate of each of 12 fish in individual chambers (Morgan and Kuhn, 1974; Morgan, 1977). Data gathered from each fish over a 5-day period are used to set a 99 percent upper confidence limit on the ventilatory rate for that fish. A voltage level proportional to this limit is then set into a device which activates an alarm light should any fish exceed its confidence limit. With a criterion for toxicant detection set as the response of at least 60 percent of the fish, a wide variety of materials were detected within 2 to 4 hours at levels of 5 to 10 percent of their 48-hour LC₅₀s. Toxicants tested included cadmium, copper, magnesium, lead, mercury, phenol, ammonia, cyanide, carbamate, chlordane, parathion, and pentachlorophenol.

Using a similar system with rainbow trout, Sloof (1978) found that for 12 organic and inorganic compounds, at least three of four exposed fish responded within 24 hours to concentrations ranging from 1 percent (cadmium) to 30 percent (acrylonitrile, cyanide, lindane) of the 48 hour LC₅₀ of the zebra fish (Brachydanio rerio). The corresponding LC₅₀s for trout were not determined.

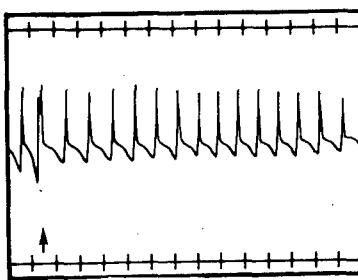
A comparison between automated monitoring systems utilizing either the opercular movement rates or activity levels of the largemouth bass (Micropterus salmoides) showed that for toxicants causing physiological impairment, the opercular rate system detected materials sooner and at lower levels. In contrast, two toxicants known to cause olfactory stimulation were detected at lower levels by the activity monitor (Morgan, 1979). As Morgan points out, it may be better to monitor multiple parameters to increase the usefulness of a toxicity early warning system.



a. OPERCULAR MOVEMENTS



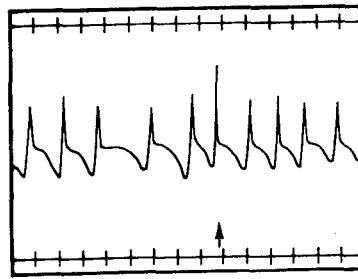
d. WHOLE BODY MOVEMENT



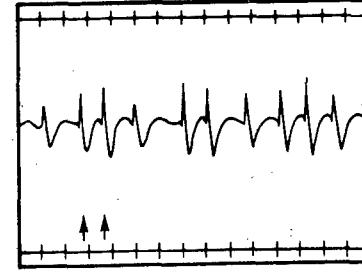
b. HIGH FREQUENCY COUGH



e. PECTORAL FIN MOVEMENT



c. SPIKE COUGH



f. OPERCULAR MOVEMENTS

Figure 1. Typical bluegill ventilatory patterns (van der Schalie, 1980).

FIELD TEST OF A SYSTEM BASED ON CHANGES IN FISH VENTILATORY PATTERNS

One of the few on-line toxicity monitoring systems to be tested at an actual industrial site was located at Celanese Fibers Company Plant on the New River in Virginia. Cellulose acetate fibers are produced at this plant, creating a process wastewater flow of approximately $0.086 \text{ m}^3/\text{sec}$. This amounted to 0.1 percent of the total river flow, based on daily averages over the 6-month period during which the monitoring system was in operation. The discussion below is based on van der Schalie et al. (1979) and more details on the design and operation of the system may be found in this reference. The manufacturing process waste has three main

components: direct process wastes; overflow from settling ponds, where waste heavily laden with cellulose acetate flakes is pumped; and sanitary sewage generated by the 1500 employees of the plant. The first two components flow together into an equalization pond with a retention time of approximately 2 days. This retention time prevents rapid changes in waste composition which may be detrimental to the effectiveness of a subsequent biologic treatment stage. On leaving the equalization pond, the waste stream is joined by the sanitary sewage, which has received primary treatment in an Imhoff tank. The waste then flows into a large aerated lagoon where microbial degradation of the largely organic waste produces an effluent of acceptable quality for direct discharge into the New River. Substantial upgrading of the wastewater treatment facilities has occurred since 1976, when the monitoring system was tested. The fish monitoring system received waste from two points in the treatment process. One type of wastewater was sampled prior to entering the equalization pond (influent wastewater), and the other was sampled as it was discharged into the New River (effluent wastewater).

Monitoring of wastewater quality of both influent and effluent wastes at the Celanese plant was carried out at the waste treatment building on the plant ground. Waste composition was monitored by 24-hour refrigerated composite samplers and continuous flow-through sensors. The sensors measured the pH and temperature of influent wastes and the pH, temperature, and dissolved oxygen concentration of the effluent waste. The treatment building was also the site chosen for the location of the fish monitoring system. This facility was supplied with a continuous sample of both waste streams as they entered the equalization pond (influent) and the river (effluent). The treatment building had a slight corrosive atmosphere, a great deal of heavy-duty electrical equipment, warm temperatures, and was located less than 2 km from a radio station broadcast tower. Together, these factors caused much interference with the operation of some of the monitor's electronic components. Vibrations and noise from the construction and renovation of the treatment facilities were also present at times during the monitoring system's operation.

The influent and effluent wastes tended to have low dissolved oxygen concentrations with high levels of BOD, COD, solids (mostly dissolved), and magnesium. The influent waste had higher temperature and solvent levels (primarily acetone), a lower pH, and, because it was sampled before equalization, a greater variability in composition. The effluent waste had a pH near neutrality, moderate temperatures, and negligible solvent concentrations. Much of the effluent suspended solids were bacterial floc, whereas those in the influent waste were usually cellulose acetate flakes.

Bluegills were the test fish used to monitor wastewater quality. A grab sample of effluent was of insufficient toxicity to allow computation of an LC₅₀ after 96 hours, while the influent waste

had a 96-hour LC₅₀ of about 18 percent wastewater. Fish were monitored in 30 L fiberglass tanks with stainless steel wire electrodes at each end of the tank (Figure 2). The feeder shown provided food at 0900 every day. Groups of four tanks were contained in modules (Figure 3). The diluter device provided dilution water or diluted wastewater to each set of tanks; a total of three modules (12 tanks) were used. One module received only dilution water and served as a control; the other two modules were supplied either diluted influent or effluent waste. River water could not be obtained for use as dilution water so well water was used instead. Wastewater was passed into the monitoring system as it flowed through the treatment process; the only delays were associated with small-volume settling chambers needed to remove some of the high solids content of the waste and with the replacement times required by the size of the test tanks and the rate of water flow through them. It is estimated that a persistent change in waste composition would not reach 50 percent of its ultimate level in the tanks until about 40 minutes after the change had begun.

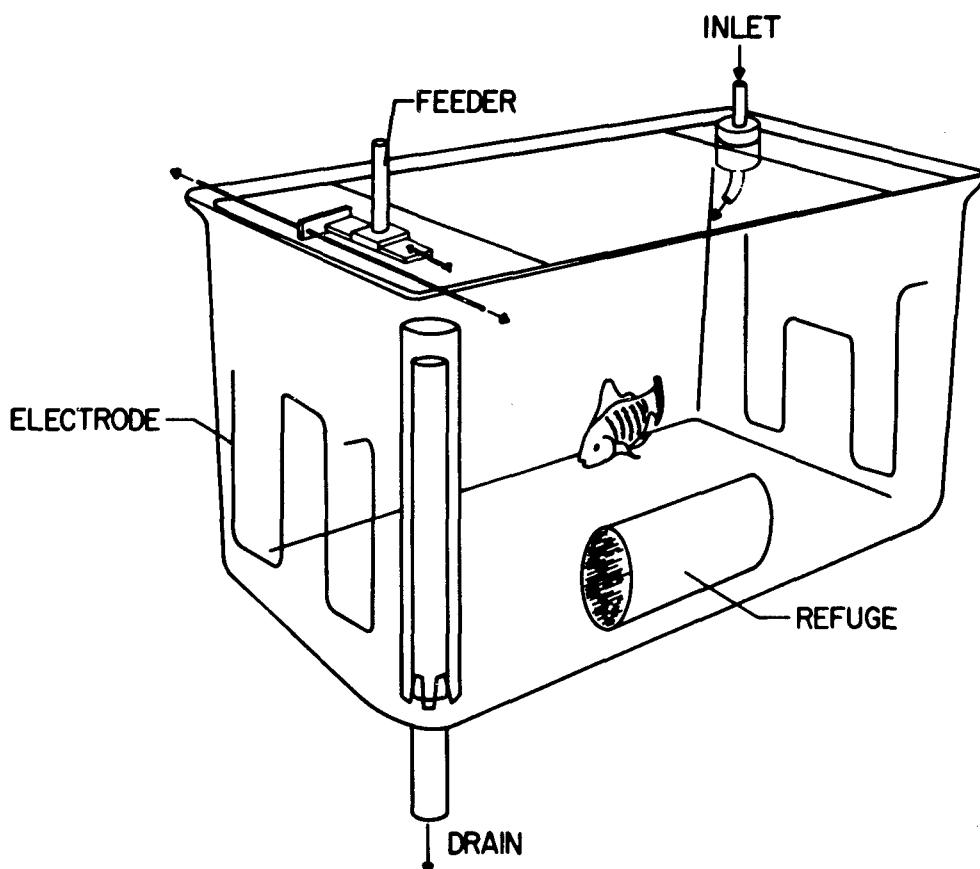


Figure 2. Tank used for monitoring fish (van der Schalie et al., 1979).

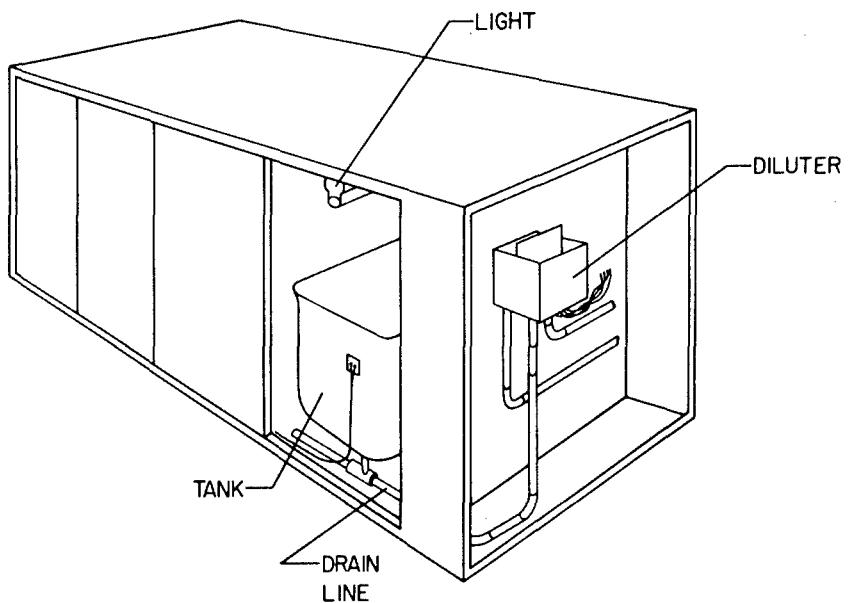


Figure 3. Module containing four tanks (van der Schalie et al., 1979).

The data pathways in the monitoring system are shown in Figure 4. The ventilatory signals from each of the 12 fish were monitored by the microcomputer at the industrial site. Every half-hour, ventilatory counts from the fish were transmitted to a minicomputer for further analysis. The microcomputer could also turn on an alarm light at the plant to indicate that the monitoring system had detected abnormal fish ventilatory patterns. The minicomputer stored the ventilatory data and printed it out at a teletype. At the start of a test, following acclimation of the fish to the chambers, several days of data were gathered during which all fish were exposed to dilution water only. These data were transmitted to the IBM 370 computer for computation of 95 percent confidence limits for each fish for each of the 48 half-hour periods in the day. These limits were then sent back and stored in the minicomputer's memory. All subsequent data from the fish, some of which were exposed to diluted wastewater, were compared to the appropriate confidence limit. When three or four fish out of the four fish in a module had ventilatory counts outside of their half-hour confidence limits, a "warning" situation was said to exist. When this occurred the minicomputer could send an immediate signal to the microcomputer, resulting in the lighting of the alarm light.

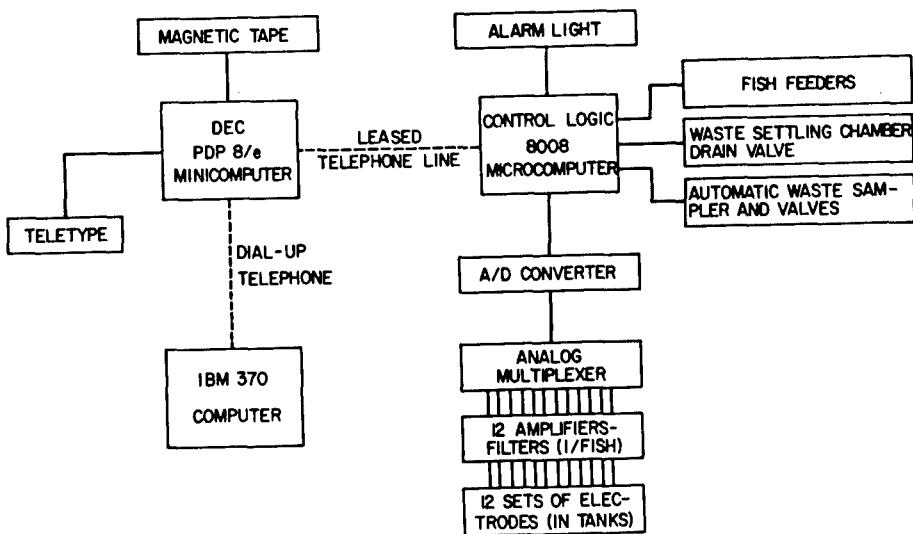
VIRGINIA TECHREMOTE INDUSTRIAL SITE

Figure 4. Data pathways for the Celanese monitoring system (van der Schalie et al., 1979).

Several factors made successful operation of the system difficult. The high solids content of the waste resulted in frequent clogging of the diluter apparatus in spite of the waste settling chambers. Electrical interference and deterioration of electrical contacts of the fish amplifiers required frequent cleaning and adjustment of the on-site electronics. These factors combined to produce a large number of "false alarms" from the fish. Although no known toxic waste spills occurred during the trial period, a simulated spill was conducted with acetone, one of the solvents commonly used at the plant. For this test, one module of four fish served as controls while the other modules both received a 5 percent effluent waste dilution to which acetone was added two times within a 5-day period. The results are shown in Figures 5 and 6. "E1" and "E2" refer to the two modules of fish receiving waste. Control fish produced only one warning during the 5-day test period. It is important to note that during the acetone exposures, the E1 group of fish had only three working amplifiers; thus all three fish would have had to respond to constitute a warning. None of the acetone levels in the first acetone introduction (Figure 5) were above the 96-hour 100 percent survival concentration according to a static toxicity test by Cairns and Scheier (1968). No warnings could be attributed to acetone at

these levels; the E2 fish produced a few apparently unrelated warnings both before and during the exposure. The second acetone exposure began 48 hours after the first. Three of four fish in the E1 group were killed by acetone concentrations which peaked at over 14,000 mg/L; the 96-hour "no-survival" concentration was 11,500 mg/L (Cairns and Scheier, 1968). Continuous warnings were not produced by the E1 fish only because one of the three fish that died was in the chamber with the inoperational amplifier. The acetone supplied to the E2 fish had a peak value near the 96-hour LC₅₀ value of 8300 mg/L. Warnings from these fish started 1 hour after acetone introduction and continued for five more half-hour periods until the acetone concentration dropped below about 1000 mg/L.

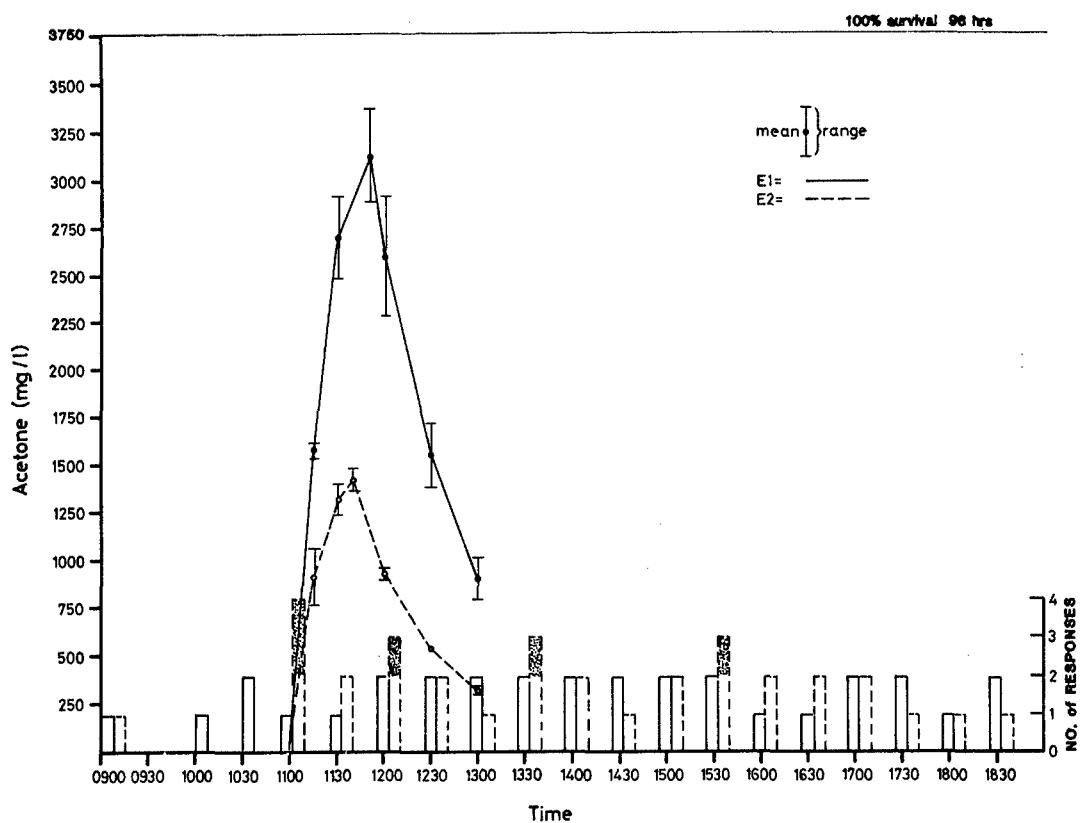


Figure 5. Response of bluegills to the first acetone addition (van der Schalie et al., 1979).

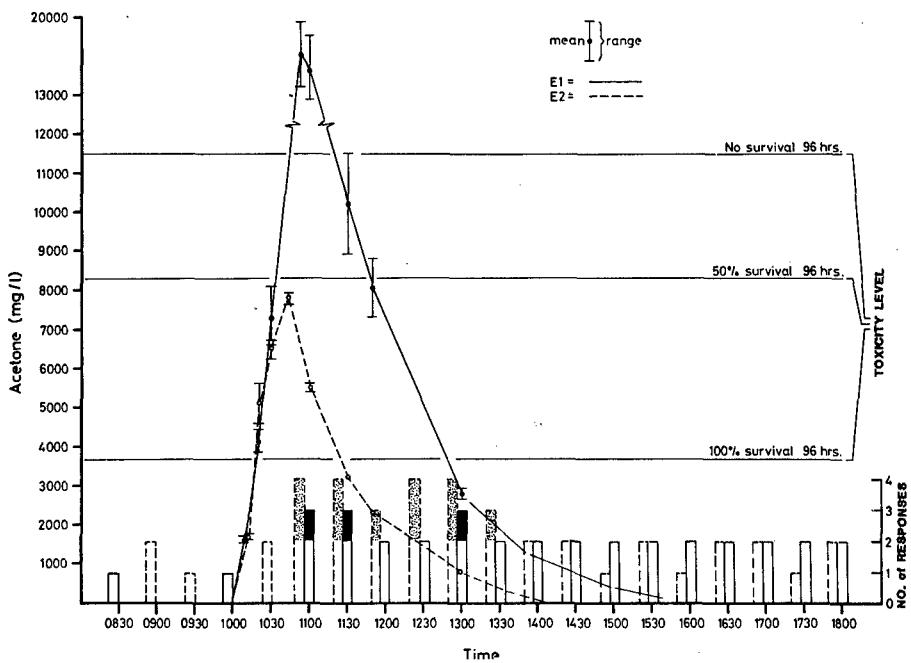


Figure 6. Response of bluegills to the second acetone addition (van der Schalie et al., 1979).

Based on the experiences with the Celanese system, a new system was constructed which avoided many of the design problems of the original system (Gruber et al., 1978, 1980). The entire monitoring system was enclosed in a portable trailer, allowing better environmental control. The three-computer monitoring system was reduced to one on-site minicomputer. The test chamber was reduced greatly in size, avoiding response lag-times associated with long retention times of large test tanks. The dilution system was also redesigned to avoid some of the clogging problems encountered in the Celanese system. The new system is now undergoing testing at the Radford Army Ammunition Plant in Virginia.

Although utilizing the ventilatory signals from fish seems to be among the more promising toxicity monitoring techniques, automated systems thus far have used only ventilatory rates to monitor fish health. As Figure 1 shows, however, much more information is available from the signal, including ventilatory depth (relative strength of the ventilatory movements is represented by peak heights), coughing rate, and the amount of large body movements. Recently, a computer program was developed to monitor all of these features (van der Schalie, 1980). The accuracy of this program compared to visual analysis of the same signals is shown in Table 2. Data were taken from 22 fish over 6 days; a total of 1173.75 minutes were recorded both by the computer and on strip chart

records for visual analysis. Visually, only 5.0 min of whole-body movements were seen. The microcomputer correctly identified 2.0 min, missed 3.0 min, and incorrectly counted 1.75 min when movement was not present. This amount of error is large in relation to total movement time determined visually, but small in relation to the size of the total data base. The movement error rate might become more significant if a particular toxicant being tested increased the activity level of the fish.

TABLE 2. SUMMARY OF VENTILATION AND COUGH ERROR RATES FOR 22 FISH*

Parameter	Visual Counts		Computer Error, Percent of Visual Count			
	Average Rate, min ⁻¹	Range of Rates Among Fish, min ⁻¹	Missed Counts	Extra Counts	Total (Extra + Missed)	Range of Extra + Missed
Ventilation Counts	19.7	12.4 to 26.5	-5.6	+4.9	-0.7	(-)0.1 to (+)40.6
Coughs	0.51	0.16 to 0.88	-52.9	+13.9	-39.0	(-)85.7 to (+)44.4

* Data taken from van der Schalie, 1980.

The overall error rates for ventilation counts were relatively small, and the missed counts and extra counts tended to cancel each other out. For two fish there were exceptionally high positive error rates (+20.3 and +40.6 percent). These occurred because the small secondary peaks found in many ventilatory signals (Figure 1f) were enlarged in these fish nearly to the height of the primary opercular peak, resulting in extra opercular movement counts. Such high error rates would not necessarily be a problem in a system designed to detect toxicant-induced changes, because relative, not absolute, changes in the ventilatory parameters would be measured. For example, as long as the microcomputer consistently records 40 percent more opercular counts than are present, it may still pick up a 10 percent increase in opercular rate caused by a toxicant.

This reasoning also applies to cough error rates. The microcomputer was programmed to identify as coughs only those peaks that most closely fit the criteria for a cough. Thus, many peaks thought to be coughs by visual inspection were missed by the microcomputer, resulting in a high negative error rate. The large positive error rate present for one fish (44.4 percent) was based on a total of only nine visual coughs. Further reductions in the cough error rates are unlikely, given the difficulty in visually defining a cough (Maki, 1979) and the large variability in ventilatory patterns between individual bluegills.

CONCLUSION

It would appear that the major factor determining the success or failure of biological toxicity monitoring systems will be their operation not in the laboratory but in actual field situations. Although industries may be initially reluctant to allow monitoring of their effluents, the benefits from an operational system would go, not only to the public, but also to the industry in terms of better public relations and perhaps even in savings of valuable process materials. Monitoring devices using fish are the most advanced at the present time, and systems which are able to track multiple instead of single response parameters would seem to offer more promise for the detection of acutely toxic waste discharges.

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**ULTRASTRUCTURAL ALTERATIONS IN FAT-HEAD MINNOWS
AFTER EXPOSURE TO JP-4**

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INTRODUCTION

There is a need for the acquisition of basic biological information regarding the consequences of exposure to petroleum-derived JP-4 aviation fuel in aquatic organisms. Due to the significant amount of JP-4 consumed by the Air Force on a daily basis (Berry, 1980), a distinct possibility exists of accidental spills and consequential seepage of fuel into the aquatic environment.

Petroleum-derived aviation fuel consists of a variety of aromatic compounds including toluene, benzene, xylene, and naphthalene. Investigators have determined that the rate of aromatic hydrocarbon accumulation in aquatic organisms is highly species specific (Neff et al., 1976). Toluene is capable of accumulating in a variety of organs once uptake has occurred (Berry and Fisher, 1979), and compounds such as benzene, xylene, and naphthalene have been analyzed as to their relative toxicities to aquatic organisms (Meyerhoff, 1975; Benvile and Korn, 1977).

There is a paucity of information regarding histopathologic and physiologic responses of aquatic vertebrates to the aromatic hydrocarbons mentioned. Investigators report that juvenile striped bass exhibit increases in respiratory rates after exposure to sub-lethal concentrations of benzene (Brocksen and Bailey, 1973). Histopathologic investigations of mummichogs exposed to sublethal

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quantities of naphthalene indicate that the toxicity is due primarily to a generalized blood stasis (DiMichele and Taylor, 1978).

The primary objective of this investigation was to determine the acute ultrastructural effects of the water-soluble fractions of petroleum-derived JP-4 aviation fuel on selected organs of the fat-head minnow, Pimephale promelas. The study was restricted to analyses of gill, pseudo-branch, nasal mucosa, and kidney excised from fish exposed to a 40 percent concentration of JP-4.

MATERIALS AND METHODS

Fat-head minnows of various weights were divided into 2 groups of 12 fish each. One group was released into a 20 gallon tank containing a 40 percent solution of the water-soluble fractions of petroleum-derived JP-4. The remaining group was maintained in a 20 gallon tank of water and functioned as a control.

At 6, 12, 24, and 48 hours after the initial exposure, 3 fish from each tank were removed and placed in a 3% glutaraldehyde-3% paraformaldehyde solution which was buffered with 0.1M sodium cacodylate (pH 7.4). To enhance the fixation of internal organs, a longitudinal slit was cut from the anus to the pectoral fins. Sections of gill, pseudo-branch, nasal epithelium, and kidney were excised and placed in vials containing cold fixative.

After a fixation period of 3 hours the tissues were rinsed in cold buffer and post-fixed in 2% osmium tetroxide for 2 hours. Subsequent to a rinse in buffer the tissues were dehydrated in a graded series of ethanol. Specimens to be analyzed by scanning electron microscopy were then critical point dried, metallically coated, and observed in an ETEC Autoscan scanning electron microscope at 20 KV.

Organs to be further processed for transmission electron microscopy were embedded in Epon 812. Thin sections were cut by an ultramicrotome and then stained with lead citrate and uranyl acetate. The tissues were examined by a JEOL 100B transmission electron microscope at 60 KV.

RESULTS

Although the ultrastructural effects of the fuel appeared to be temporally related to severity, the various manifestations of exposure were evident even at the shortest time period investigated. In addition, the exposures were determined to be sub-lethal.

GILL

Cells comprising the gill filaments and secondary lamellae were analyzed during the investigation. The surface junctional sites of adjacent control cells were raised in the form of ridges. Micro-projections of non-uniform dimensions protruded from the cellular surfaces (Plate I; Figure 1). Gill filaments and secondary lamellae of experimental organisms displayed altered surface junctional sites as manifested by the formation of blebs (Plate I; Figure 2). The micro-projections, evident on the surface of control cells, were significantly reduced, resulting in a somewhat smoother contour.

There was no evidence of a consistent or repetitive form of cellular degeneration among the gills examined. However, focal sites of surface epithelial cell degradation were noted among the secondary lamellae (Plate I; Figure 3). There was no indication of fragmentation or dissociation of plasma membranes directly contacting the fuel.

Pillar cells, whose lateral extensions form the smooth, inner surface of capillaries within the secondary lamellae, displayed no abnormalities in regard to cytoplasmic organelles. Plasma membranes lining the lumen of several capillaries appeared distended and irregular in appearance (Plate I; Figure 4). There was no indication of a disruption of the basal lamina located between the pillar and epithelial cells.

Granulocytes were present within the gill filaments and secondary lamellae; however, when compared to control tissue the concentration appeared to be within normal ranges. Chloride and mucoid cells located at the base of secondary lamellae appeared normal (Plate I; Figure 5).

PSEUDO-BRANCH

The outer surface of the control pseudo-branch displayed ridge-like extensions at the junctions of adjacent cells (Plate II; Figure 7). In addition, similar elevations of varying lengths and patterns were evident along the cellular surfaces. In experimental organisms, although the junctional ridges maintained their integrity, the cellular surface elevations appeared altered with respect to concentration and height (Plate II; Figure 6).

The primary cell type of the pseudo-branch is uniquely structured with an ordered arrangement of tubules which are tightly grouped both together and in conjunction with elongated mitochondria (Plate II; Figure 8). The tubules appear to evolve from invaginations of the plasma membrane which contacts the basal lamina positioned adjacent to the capillary systems of the pseudo-branch.

PLATE I. Explanation of Figures
Figures 1 - 5 Gill

Figure 1. Surface view of gill filaments revealing ridge-like elevations (arrowhead) which form at cellular junctions. Micro-projections (arrow) of various dimensions extend from the cellular surface. Control gill. X 9000.

Figure 2. Membranous blebs (arrowheads) protrude from the ridge-like elevations associated with the cellular junctions. In contrast to control organs there is an absence of micro-projections along the cellular surface. 48 hours subsequent to initial exposure. X 9000.

Figure 3. A surface epithelial cell (EP) of a secondary lamella appears in a state of degradation. Cytoplasmic extensions (arrowhead) of the pillar cell (PC) form the inner surfaces of the capillaries associated with the lamella. 48 hours subsequent to initial exposure. X 15,000.

Figure 4. The inner surface of the capillary within a secondary lamella is disrupted due to the formation of cytoplasmic extensions (arrowheads) and outfolds of the endothelial plasma membranes. There is no indication of ultrastructural alteration within a surface epithelial cell (EP). 24 hours subsequent to initial exposure. X 28,000.

Figure 5. Chloride (CH) and mucoid (MU) cells located at the base of secondary lamellae display no evidence of modifications or degradation. 24 hours subsequent to initial exposure. X 12,000.



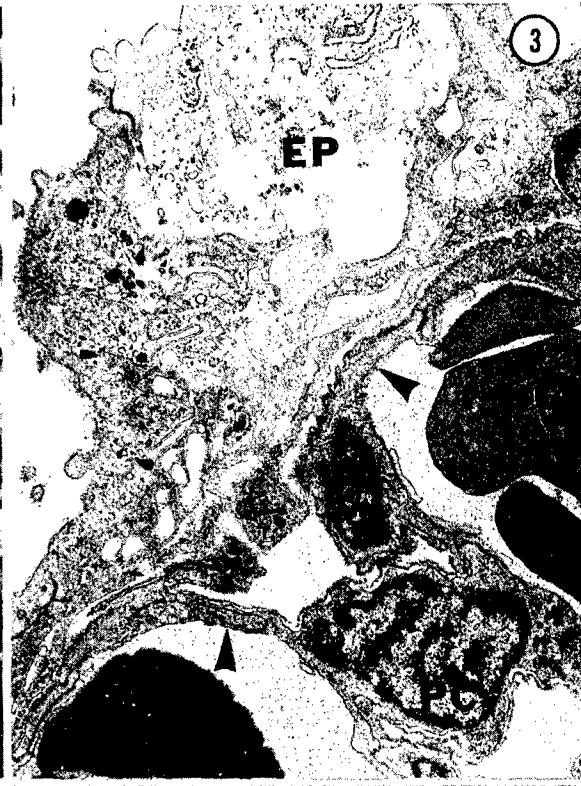
1



2



4



3



5

The fuel exerts a disruptive effect on the structured system of mitochondria and tubules. In essence, the latter organelles were unable to maintain the close association noted in control tissue (Plate II; Figures 9 and 10).

Vacuoles, both membrane and non-membrane bound, were evident in a majority of the cells examined from experimental tissue (Plate II; Figures 9 and 10). Occasional mitochondrial degradation was manifested in the form of distensions of the outer membrane, and in some instances by the development of myeloid figures. Although morphometric analyses were not conducted, random scans of pseudo-branch indicated that substantial members of cells were at least partially affected by the fuel.

PLATE II. Explanation of Figures
Figures 6 - 10 Pseudo-branch

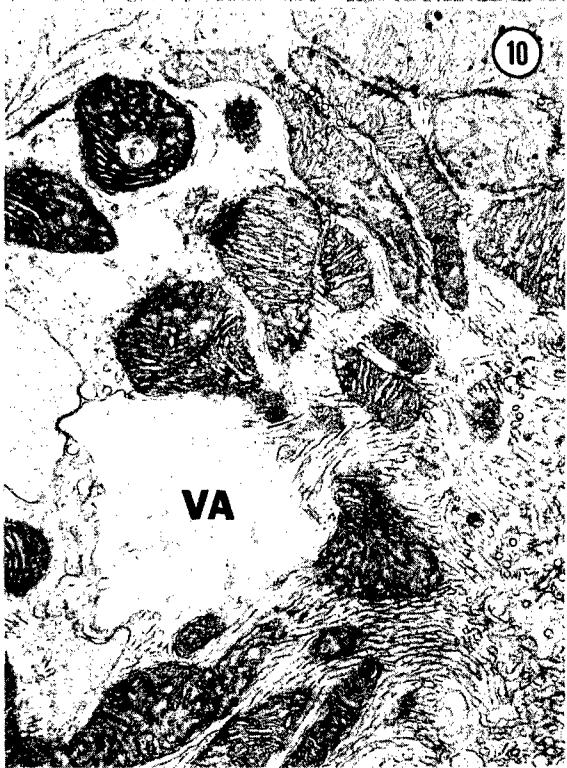
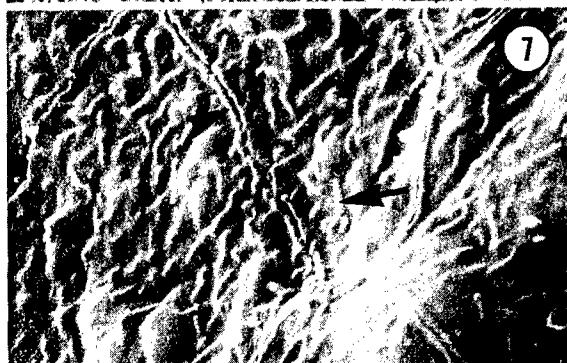
Figure 6. The ridge-like extensions associated with cellular junctions (arrowhead) are normal. However, the elevations which protrude from the cellular surfaces (arrow) appear to have increased in concentration, and in certain instances elevation. 6 hours subsequent to initial exposure. X 2000.

Figure 7. Surface view of pseudo-branch depicting ridge-like arrangement of cellular junctions (arrowhead). Plasma membranes of cells elevate at various sites to form irregularly shaped ridges (arrow). Control pseudo-branch. X 2000.

Figure 8. The predominant cell of the pseudo-branch is characterized by a structured arrangement of tubules, which are tightly grouped together and in conjunction with mitochondria (arrowhead). Control pseudo-branch. X 18,000.

Figure 9. The formation of vacuoles (VA) among cells which line the capillaries (CA) of the pseudo-branch results in a disruption of the tubule-mitochondria complex evident in control cells (see Figure 8). 6 hours subsequent to initial exposure. X 10,000.

Figure 10. A non-membrane bound vacuole (VA) exerts a disruptive effect upon the association of mitochondria and tubules. 6 hours subsequent to initial exposure. X 28,000.



NASAL MUCOSA

Surface projections of the cells comprising the nasal mucosa were present in the form of cilia and microvilli. Compared to the control (Plate III; Figure 11), cellular projections of experimental tissue exhibited no discernible alterations (Plate III; Figure 12). Minute blebs were noted on the surface of cilia in experimental organs; however, similar membranous protrusions were also observed on the surface of cilia in control groups. The integrity of plasma membranes directly contacting the fuel was maintained. The underlying epithelial cells of the control nasal mucosa were characterized by cytoplasms of varying electron-densities; however, no evidence of cellular degradation was noted (Plate III; Figure 13).

Cellular abasement, manifested by the formation of myeloid figures, electron-dense bodies, and organelle disruption, was pronounced throughout the epithelial region of experimental tissue (Plate III; Figure 14).

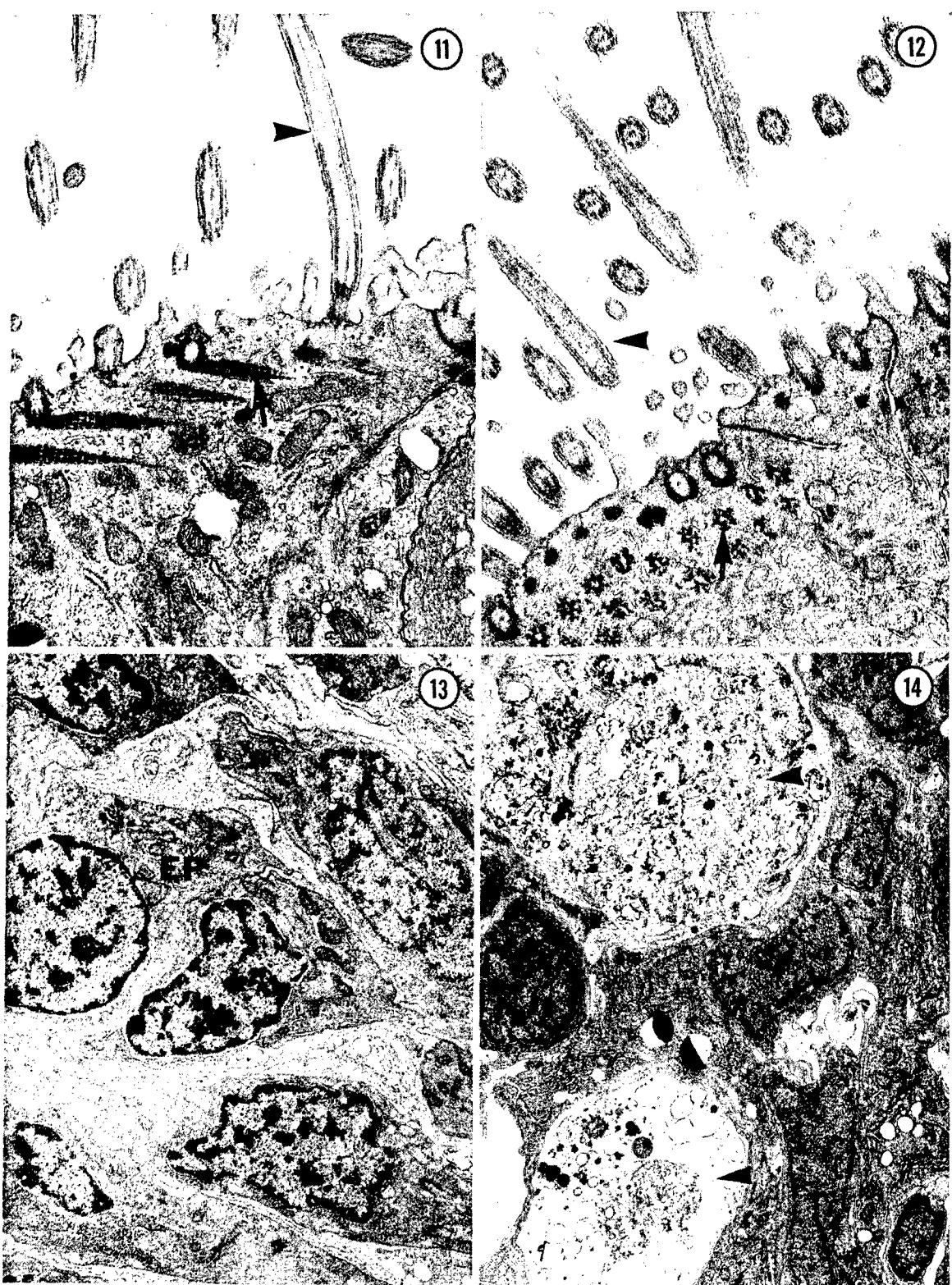
PLATE III. Explanation of Figures Figures 11 - 14 Nasal Mucosa

Figure 11. Cilia (arrowhead) protrude from the surface of the nasal mucosa. The structures are anchored to the periphery of the cell by rootlets (arrow). Control nasal mucosa. X 28,000.

Figure 12. The outer plasma membranes of cilia (arrowhead) distended to form minute blebs. The rootlets (arrow) viewed in cross-section display no signs of dissociation or alteration. 6 hours subsequent to initial exposure. X 30,000.

Figure 13. Cells (EP) comprising the nasal mucosa differ in shape and electron density. There is no indication of cellular degradation. Control nasal mucosa. X 15,000.

Figure 14. Cellular degeneration (arrowhead) is prominent among the cells positioned deep in the mucosa. Alteration is characterized by a disruption of cytoplasmic organization and a proliferation of vacuoles. 24 hours subsequent to initial exposure. X 12,000.



KIDNEY

Cells comprising the proximal convoluted tubules of experimental fish appeared to possess a greater number of apical vacuoles than the control (Plate IV; Figures 15 and 16). No ultrastructural alterations were detected among the cells and basal lamina associated with the glomeruli (Plate IV; Figure 17).

Endothelial cells normally adjacent to the basal lamina of proximal and distal convoluted tubules were frequently observed in a state of dissociation. Plasma membranes of the aforementioned cells appeared fragmented with a resulting exposure of the basal lamina to the peritubular vessels. At the sites of alteration some disruption of the basal lamina was noted (Plate IV; Figure 18).

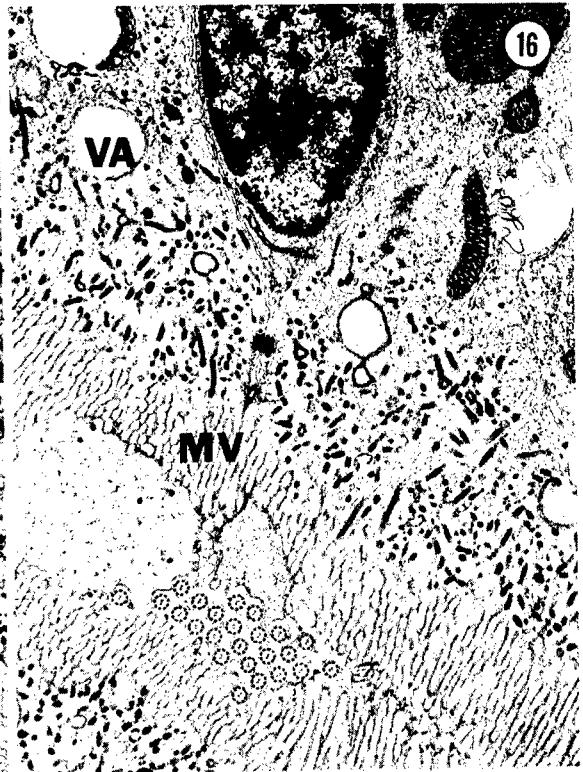
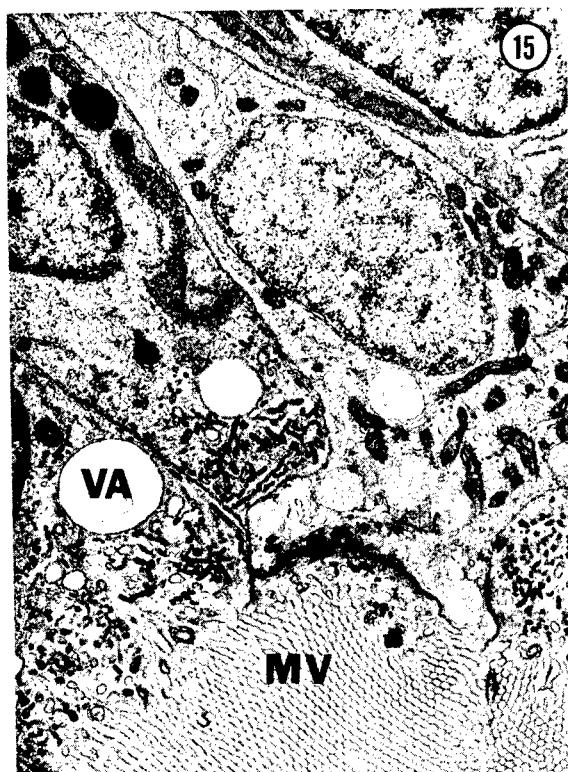
PLATE IV. Explanation of Figures Figures 15 - 18 Kidney

Figure 15. Cells of the proximal convoluted tubule are characterized by a predominance of microvilli (MV) and apical vacuoles (VA). Control kidney. X 15,000.

Figure 16. There is no evidence of ultrastructural disruption among the cells comprising the proximal convoluted tubule. Microvilli (MV) are normal in configuration; however, there appears to be an increase in the concentration of apical vacuoles (VA). 48 hours subsequent to initial exposure. X 17,000.

Figure 17. Pedicels (arrowhead) associated with the basal lamina of experimental glomerular capillaries display no irregularities. All leukocytes (LE) examined appear normal. 24 hours subsequent to initial exposure. X 15,000.

Figure 18. The basal lamina (arrowhead) associated with the epithelial cells (EP) of the proximal convoluted tubule exhibits focal sites of disruption. 48 hours subsequent to initial exposure. X 38,000.



DISCUSSION

Results from the present investigation indicate that the water-soluble fractions of petroleum-derived JP-4 fuel at the 40 percent exposure level induce sub-lethal, ultrastructural alterations among the tissues examined. Within each tissue the effects appear to be reproducible and predictable. The ultrastructural integrity of plasma membranes directly contacting the fuel is maintained. It appears that the aromatic hydrocarbons do not exert their observable effects upon biological membranes in the capacity of an organic solvent.

Based strictly upon ultrastructural observations, the physiologic and biochemical capabilities of damaged cells are difficult to ascertain. Although cellular degradation is evident in all the experimental tissues examined, the ultrastructural effects on the gills and kidney appear localized. Thus the latter organs presumably maintain their functional capabilities. Because the disruptive nature of the fuel is more comprehensive in regard to the nasal mucosa and pseudo-branch, determinations on the extent of physiologic impairment are more tenuous.

The significance of the dissociation of mitochondria-tubule complexes present in cells of the pseudo-branch is difficult to resolve since the latter organelles basically appear normal in structure. The disruptive process is noted in association with vacuole formation and in its absence. It is probable that the close proximity of mitochondria to the tubular network is required for a particular biochemical process, and that the disruptive nature of the fuel would adversely affect normal functions. There were relatively few observations of cells in the latter stages of degradation which is usually characterized by the presence of multiple myeloid figures and massive disruption of organelles.

Cellular degradation is pronounced in the deep epithelial layers of the nasal mucosa. Although lesions in this area would not prove lethal, the degeneration may significantly impair the ability of the fish to respond to chemicals within the aquatic environment.

The phenomenon of an elimination of micro-projections among cells that comprise the surface of the gill would indicate a dramatic response by the latter organ to the presence of a potentially toxic chemical in the environment. Within the secondary lamellae of gills, focal sites of plasma membrane alteration are evident along the lining of capillaries. The effect of the latter on functional capabilities would not appear to be pronounced since the lesions are not extensive. Occasionally, granulocytes are present

in the secondary lamellae, but the same cellular arrangement is observed in control tissue. Plasma membranes forming the surface of gill filaments and lamellae apparently are unaffected structurally by direct contact with the fuel.

Acute JP-4 exposures to the kidney appear to alter selectively the endothelial cells associated with the distal and proximal convoluted tubules. This phenomenon indicates a lack of uniformity in regard to the effects of JP-4 on endothelial cells as a whole. Comparable cells of the pseudo-branch and gills display little susceptibility to the fuel.

In summary, the water-soluble components of JP-4 at the 40 percent exposure level are capable of inducing ultrastructural alterations in the organs selected for the present investigation. The effects span a wide spectrum of observable alterations including the modification of surface structures, the degradation of cells, the proliferation of vacuoles, and a disruption of the structured association of cellular organelles.

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OPEN FORUM

MAJ. LIVINGSTON (Air Force Aerospace Medical Research Laboratory): Dr. Bishop, do you have a matrix of testing for your fish assessment system that you use or is it random testing for whatever happens to be available in the laboratory? If you do have a matrix of testing, is it the same for each class of chemical?

DR. BISHOP (The Proctor and Gamble Company): No, one of the things that I disagree with vehemently is the checklist approach to environmental safety testing. What I've tried to outline for you this morning is my belief that a program for assessing safety has to be developed specifically for the chemical of question based on its physical/chemical properties. Without that, the whole thing just degenerates into performing every test for every organism. We're somewhere in the middle. There are those people who say the only way to establish safety is to run every test for every organism. You can't do it. On the other hand, you often have the product development people who say run an LC₅₀ and tell me that it's safe. I'm somewhere in the middle. In the programs that we're involved with typically day to day, every one is different.

MAJ. LIVINGSTON (Air Force Aerospace Medical Research Laboratory): Dr. Bourquin, have you looked at the effect of the binding capacity of different cores on the microbial degradation?

DR. BOURQUIN (United States Environmental Protection Agency): Yes, we have a separate ongoing study where we're actually determining the relationship of microbial activity both from heterotrophic activity levels and total biomass in relationship to binding using a number of different pesticides. I've referred to binding as a microbiologically mediated process and not sorption, sorption being primarily mediated by the total organic content in that particular system. We haven't really discovered a real solid relationship between the level of heterotrophic activity in microorganisms or total biomass and the binding capacity. It's probably a combination of both activity level and the specificity of the enzymes involved in that particular process that binds the chemical to the sediments such as, for example, the methylparathion conversion to aminomethylparathion which is actually the compound that's probably being bound.

DR. DEETER (U. S. Army Environmental Hygiene Agency): In reference to kepone in the James River, it's my understanding that the kepone is disappearing from the water and the fish at a much higher rate than predicted. If that's true, what's actually happening to the kepone?

DR. BOURQUIN (United States Environmental Protection Agency):

I'm sure the kepone is being rapidly sorbed by the biological components as well as the scouring of sediments from the area. Levels of kepone are unmeasurable in the water column per se, it's sorbed to particulates in that system. I think most of it is being removed biologically. There is no evidence to date that shows there should be any significant microbial metabolism of kepone in the James River or in the laboratory.

DR. HODGE (University of California, San Francisco): You showed us a graph comparing pretreatment of water with paranitrophenol and nontreated water. In this graph the ordinate was labeled % of theoretical PNP. What is the theory?

DR. BOURQUIN (United States Environmental Protection Agency): We've treated the systems with either ¹⁴C-labeled paranitrophenol or labeled methylparathion, whichever the case may be, and the percent theoretical refers to theoretical carbon, or the percentage evolved and theoretical carbon dioxide expected from the mineralization of that compound. In other words, a percentage of the total carbon 14 labeling released in that system.

DR. HODGE: I believe that in every one of those tests described the PNP pretreatment was much more effective in breaking down or evolving CO₂ than the methylparathion?

DR. BOURQUIN: Correct. The paranitrophenol is the actual compound that undergoes microbial degradation or transformation by a specific enzyme system. The conversion of methylparathion to paranitrophenol is a hydrolysis reaction which also is mediated both chemically and microbiologically by nonspecific hydrolysis.

MR. VERNOT (University of California, Irvine): Has anybody considered using genetic engineering to attack the problem of microbial degradation of the complex chlorinated hydrocarbons - perhaps by doing a screening study for micro-organisms that might have the capability of degrading material and then interchanging the genetic material through plasmid techniques genetic engineers use now, transferring that ability to an organism native to the contaminated area and possibly in that way cleaning up the contamination?

DR. BOURQUIN (United States Environmental Protection Agency): As you know, genetic engineering is probably one of the hottest issues in microbial science today and there is a lot of work going into developing microbial strains capable of degrading a large area of compounds. The genetic capability for dechlorinating and degrading complex chlorinated hydrocarbons really doesn't exist that predominantly in nature and we don't have a genetic handle on dechlorination reactions except to a very limited extent on mono- and dichlorinated compounds. Engineering an organism capable of dechlorinating a compound would be rather difficult under those

circumstances. The second portion of your question in putting an organism like that back into the environment again brings up another problem. We would have great difficulty with that based on the fact that most of these genetically engineered organisms are not that stable and probably could not compete in the natural environment with indigenous populations. That is particularly true for treatment of the very low levels of compounds that we're talking about in the environment.

MR. VERNOT: My suggestion was not that you use the organism that you isolated, but to transfer the genetic material from that organism to one that was occurring naturally in the environment.

DR. BOURQUIN: The fact that you transfer that genetic information to the organism does not necessarily mean that that organism will be able to use that genetic information in the environment and that is due to the competition within the environment. If an organism has 100 times more readily available and easily degradable substrate than the low level of pollutant in that particular environment, it has no reason to use that low level pollutant which probably requires more energy to degrade.

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TECHNICAL REVIEW AND APPROVAL

AFAMRL-TR-81-149

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER



ROGER C. INMAN, Colonel, USAF, BSC
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